Universidade Nova de Lisboa

Instituto de Higiene e Medicina Tropical

Unraveling of *Borrelia burgdorferi* sensu lato genospecies diversity in Portugal towards the development of more efficient diagnostic tools for Lyme disease

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Thesis presented to obtain the Ph.D. Degree in Biomedical Sciences, specialization Microbiology

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To my brother and parents

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Abstract

Ixodids (hard-body ticks), are the most important vectors of pathogenic agents, responsible for emerging diseases like Lyme disease (LD). This zoonosis is caused by spirochetes of *Borrelia burgdorferi* sensu lato (s.l.) complex, transmitted by *Ixodes ricinus* tick, the main vector in Europe. LD is a multisystem disorder with different clinical presentations, and a complex diagnosis. In Portugal is still underdiagnosed and the notification, although mandatory, is scarce.

The main goal of this research was to develop molecular protocols, namely a *TaqMan* real-time PCR algorithm and an isothermal amplification, for the identification of the most prevalent genospecies of *B. burgdorferi* s.l. in Portugal. The development of this goal, also allowed to evaluate the bio-ecological characteristics of the ixodofauna in nine districts of the country (north, center and south), where the presence of *I. ricinus* tick was previously reported, and also to determinate *B. burgdorferi* s.l. infection rate in the vector and hosts. The obtained results are presented in 10 scientific papers, from which seven are published, two in submission and one in preparation.

Of all the achieved results, is important to highlight the variations in the distribution of ticks possible into new regions, probably related to changes in the landscape, climate and vegetation, to which ticks are very sensitive. Moreover, several *B. burgdorferi* s.l. species were detected in ticks, apart from those commonly recognized as vectors. *B. lusitaniae* species was the most prevalent species in the vector, which was collected in six of the nine selected districts. Unexpectedly, during this study, three species from Relapsing Fever *Borrelia* complex were identified in questing ticks, namely *B. miyamotoi* in an *I. ricinus* nymph, and two possible new Relapsing Fever like-*Borrelia* in *Haemaphysalis punctata* and *Rhipicephalus sanguineus* tick species.

Alongside, *B. burgdorferi* s.l. DNA was identified in biological samples from pets (dogs and cats) and sylvatic animals (wild boars), confirming the importance of these animals, mostly pets, as sentinel for early detection of emerging LD, helping to access the risk of *B.*

burgdorferi s.l. spirochetes transmission to humans and other animals with economic importance (e.g. cattle), in restricted geographical areas.

It was also possible to optimized two molecular protocols for LD laboratory diagnosis, one of which, a *TaqMan* real-time PCR algorithm allowing the identification of four of the most prevalent species of *B. burgdorferi* s.l. in Portugal, presenting high sensitivity and specificity, and contributing for a more accurate diagnosis of LD.

Some of the subjects introduced and developed in this thesis deserve more detailed investigation. However, this work alerts for the introduction of possible 'new' Relapsing Fever *Borrelia* species in questing hard-body ticks, whose pathogenicity is still unknown, but it may become a risk to public health; contributes to the spatial update of important areas for LD eco-epidemiology in Portugal; and innovates in the molecular diagnosis of this zoonosis, being a valuable tool to clinicians, allowing a more accurate therapy of the patients.

Keywords: *B. burgdorferi* s.l. eco-epidemiology, *B. miyamotoi*, "new" *Borrelia* species from Relapsing Fever complex, LD molecular diagnosis.

Resumo

Ixodídeos (carraças de corpo-duro), são importantes vetores de agentes patogénicos, responsáveis por doenças emergentes como a doença de Lyme (DL). Esta zoonose é causada por espiroquetas do complexo *Borrelia burgdorferi* sensu lato (s.l.) transmitidas por carraças da espécie *Ixodes ricinus*, o principal vetor na Europa. A DL é uma doença multisistémica com diversas apresentações clínicas e diagnóstico complexo. Em Portugal é ainda pouco diagnosticada e a notificação, apesar de obrigatória, é escassa.

A presente investigação teve como principal objetivo desenvolver ferramentas moleculares, nomeadamente um algoritmo de PCR em tempo real e uma amplificação isotérmica, para identificar as espécies de *B. burgdorferi* s.l. mais prevalentes em Portugal. O desenvolvimento deste objetivo permitiu também avaliar as características bioecológicas da ixodofauna presente em nove distritos do país (norte, centro e sul) nos quais o vetor *I. ricinus* havia sido anteriormente reportado, e ainda determinar a taxa de infeção por *B. burgdorferi* s.l. no vetor e hospedeiros. Os resultados obtidos são apresentados sob a forma de artigos científicos (dez), dos quais sete estão publicados, dois em submissão e um em preparação.

Do conjunto dos resultados alcançados, importa realçar as variações observadas na distribuição das carraças para possivelmente para novas regiões, provavelmente relacionadas com alterações ao nível da paisagem, clima e vegetação, às quais as carraças são muito sensíveis. Acresce o facto de várias espécies de *B. burgdorferi* s.l. terem sido detetadas em carraças que não aquelas até agora reconhecidas como vetores. A espécie *B. lusitaniae* foi a mais prevalente no vetor, estando este presente em seis dos nove distritos selecionados. Surpreendentemente no decurso deste estudo, identificaram-se três espécies do complexo *Borrelia* recorrente em carraças da vegetação, nomeadamente, *B. miyamotoi* numa ninfa *I. ricinus*, e duas possíveis 'novas' espécies do complexo *Borrelia* recorrente em carraças da espécie *Haemaphysalis punctata* e *Rhipicephalus sanguineus.*

Paralelamente, foi identificado DNA de *B. burgdorferi* s.l. em amostras biológicas de animais de estimação (cães e gatos) e de animais silváticos (javalis), confirmando a importância destes animais, principalmente os de estimação, enquanto sentinela para uma deteção precoce

da DL, ajudando na determinação do risco de transmissão das espiroquetas de *B. burgdorferi* s.l. a humanos e outros animais com importância económica (ex. bovinos), em áreas geográficas restritas.

Foi também possível otimizar dois protocolos moleculares para o diagnóstico laboratorial da DL, um dos quais, um algoritmo de PCR em tempo real que permite a identificação de quatro das espécies de *B. burgdorferi* s.l. mais prevalentes em Portugal, apresentando elevada sensibilidade e especificidade e contribuindo para um diagnóstico mais preciso da DL.

Alguns dos aspetos introduzidos e explorados nesta tese necessitam ainda de uma investigação mais detalhada. No entanto, este trabalho alerta para a introdução de possíveis 'novas' espécies do complexo de *Borrelia* recorrente em carraças de corpo-duro da vegetação, cuja patogenicidade é ainda desconhecida, mas que poderão tornar-se um risco para a saúde pública; contribui para a atualização espacial de áreas importantes na eco-epidemiologia da DL em Portugal; e inova no diagnóstico molecular desta zoonose, constituindo um valioso suporte para os clínicos, permitindo uma terapêutica mais direcionada dos doentes.

Palavras-chave: Eco-epidemiologia de *B. burgdorferi* s.l., *B. miyamotoi*, 'novas' espécies de *Borrelia* do complexo da Febre Recorrente; diagnóstico molecular da DL.

List of Abbreviations

ACA – Acrodermatitis Chronica Atrophicans

ARS – Administração Regional de Saúde

bDNA – branched DNA

bdr gene – *Borrelia* direct repeat gene

BmpA – laminin-binding protein

BSK II – Barbour–Stoenner–Kelly-II medium

BSK-H – Barbour–Stoenner–Kelly modified medium

CDC – Centers of Disease Control and Prevention

CEVDI – Centro de Vectores e Doenças Infecciosas, INSA (= Centre for Vectors and Infectious Diseases Research, INSA)

CL – Control Line

CO_2 – Carbon dioxide

COXII – Cytochrome c oxidase subunit II

CSF – Cerebrospinal Fluid

CWD – Cell wall deficient

DEET - N,N-diethl-meta-toluamide

DGS –Direção-Geral de Saúde (=Directorate-General of Health)

DL – d-loop

dLAMP – Duplex LAMP

DNA – Deoxyribonucleic Acid

DraI – Restriction enzyme from *Deinococcus radiophilus*

Ds – Double-stranded

EIA – Enzyme Immunoassay

ELISA - Enzyme-Linked Immunosorbent Assay

EM – Erythema migrans

EUCALB – European Concerted Action on Lyme Borreliosis

FDA – USA Food and Drug Administration

Fe – Iron

FRET – Fluorescence Resonance Energy Transfer

IDSA – Infectious Diseases Society of America

List of Abbreviations (Cont.)

IFA – Indirect Immunofluorescence Assay

IgG – Immunoglobulin G

IgM – Immunoglobulin M

INSA – Instituto Nacional de Saúde Dr. Ricardo Jorge (= National Health Institute Doutor Ricardo Jorge)

LAMP – Loop-Mediated isothermal Amplification

LAR – Lymphangitis-Associated Rickettsiosis

LB – Lyme borreliosis

LCR – ligase chain reaction

LD – Lyme disease

LFS – Lateral Flow Strip

LNB – Lyme neuroborreliosis

LTV – Lisboa Tagus Valley

MGB – Minor Groove Binder

MKP – Kelly–Pettenkofer medium

MLST – Multilocus Sequence Tying

Mn – Manganese

MseI – Restriction enzyme from *Micrococcus* sp.

NAATs – Nucleic Acid Amplification Tests

NIAID – National Institute of Allergy and Infectious Diseases

Osp – Outer surface proteins

OspA – Outer surface protein A

OspC – Outer surface protein C

p66 gene – *Borrelia burgdorferi* integrin ligand gene

PCR – Polymerase Chain Reaction

qPCR – quantitative real-time PCR

rDNA – Ribossomal DNA

RecA – Recombinase essential for the repair and maintenance of DNA

REP – Reptile-associated *Borrelia*

RF – Relapsing Fever

RFB – Relapsing Fever *Borrelia*

List of Abbreviations (Cont.)

RFLP – Restriction Fragment Length Polymorphism

RML – Rocky Mountain Laboratories

rpoB gene – RNA polymerase gene

RT – Reverse Transcriptase

Salp15 – Soluble cysteine-rich tick saliva protein

s.l. – sensu lato

SNPs – Single Nucleotide Polymorphisms

s.s. – sensu stricto

STTT – Standardized 2-tier testing

Taq – *Thermus aquaticus*

TBRF – Tick-Borne Relapsing Fever

Th1 – T-helper type 1

Th2 – T-helper type 2

TIBOLA – Tick-borne lymphadenopathy

TL – Test Line

TLRs – Toll-Like Receptors

TOT – Transovarial Transmission

TROSPA – Tick Receptor for Outer Surface Protein A

USA – United States of America

VlsE – Vmp (variable membrane protein)-like sequence, Expressed

WB – Western Blot

WCS – Whole-Cell Sonicate

WHO – Word Health Organization

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Chapter 5

Chapter 1

State of the art

Chapter 1 – State of the art

1.1 – Lyme disease: historical review

The name *Borrelia* was first used in 1907 by a Dutch bacteriologist, Nicholas Swellengrebel, when he named the genus after Amatée Borrel, whose descriptions about the peritrichous coat of *Spirillum gallinarum*, now designed as *Borrelia gallinarum*, formed the basis to distinguish between this genus and other spirochetes (Wright, 2009). Borrel recognized that *S. gallinarum* was very similar to Dr. Otto Obermeier spirochete found in 1873, associated with louse-borne relapsing fever. These findings were also confirmed and supplemented by the observations of the Russian physician Gregor Münch, in 1874. First named *spirocheta recurrentis* by Lebert in 1874, and then *Spirocheta obermeiieri* in 1875, the relapsing fever microorganisms were eventually also placed in *Borrelia* genus created by Dr. Swellengrebel, with the aim of creating a group that could include all arthropod-borne spirochetes (Burgdorfer, 2001).

Two years later, in 1909, Arvid Afzelius described for the first time the Erythema migrans (EM), like a skin lesion that he believed to be caused by a tick bite (Johnson et al., 1984). However, it was only in 1977 that Allan Steere and collaborators reported an outbreak of rheumatoid arthritis cases occurred among children from Lyme village, Connecticut (United States of America - USA), during the summer and early autumn. The arthritis was preceded by an erythematous skin lesion in some patients, similar to the erythema migrans previously described by Afzelius. Several possible causes were considered by the researchers, however, cultures of samples from joints and synovial fluid were negative for agents known to cause arthritis, and the causative agent of this pathology remained unknown. Nevertheless, a common fact associated to most of the children was that they lived and played near wooded areas, and the researchers started to focus their attention on deer ticks and in a possible pathogenic agent transmitted by one of these arthropods (Borchers et al., 2014). Later on the researchers began describing the signs and symptoms of this new disease, and named it Lyme Arthritis, to help the physicians to diagnose patients (Steere et al., 1977a). However, over time, some patients started also to developed neurological and cardiac abnormalities, so a complex multisystemic disorder was

recognized and the name was changed from Lyme Arthritis to Lyme disease (LD) (Steere et al., 1977b).

In 1981 the researchers from Rocky Mountain Laboratories (RML) in Hamilton, Montana that belonged to the National Institute of Allergy and Infectious Diseases (NIAID), identified the cause of LD and discovered the connection between the deer tick and the disease. The microbiologist Dr. Willy Burgdorfer, a NIAID scientist studying Rocky Mountain spotted fever, also caused by a tick bite, wondered whether the European rash, the erythema migrans, and LD might have the same cause. Along with his RML colleague physician Alan Barbour, Dr. Burgdorfer continued to study spiral-shaped bacteria, or spirochetes, from infected deer ticks. In November 1981, the two scientists found that these spirochetes caused both LD and EM, and in 1982 Burgdorfer and collaborators managed to isolate for the first time this spirochete, later named *Borrelia burgdorferi* in honor of Dr. Burgdorfer's role in its discovery (Burgdorfer et al., 1982) (Figure 1).

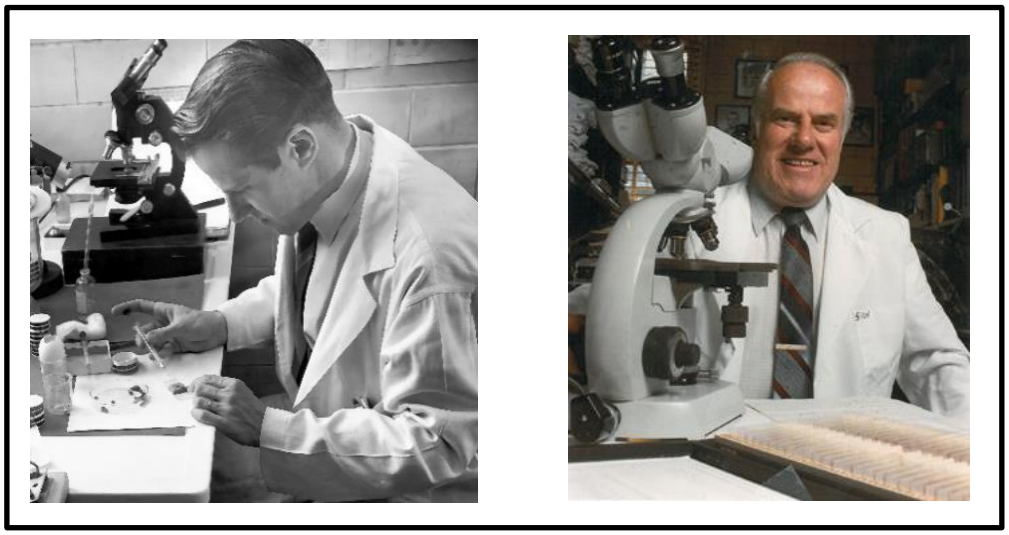


Figure 1 - Dr. Willy Burgdorfer isolates for the first time Lyme disease agent.
(Source: http://www.latimes.com/local/obituaries/la-me-willy-burgdorfer-20141121story.html; http://ravallirepublic.com/news/local/obituaries/article_b3df0fea-7058-11e4-99d6-07039cc768d3.html).

The spirochete was isolated from a deer tick, named *Ixodes dammini*, now known as *I. scapularis* and considered vector of these bacteria in USA (Steere et al., 1979). Later on the successful isolation of these spirochetes from skin, blood and cerebrospinal fluid

(CSF) samples of Lyme patients, served as the last proof of the association between these spirochetes and LD (also now called Lyme Borreliosis) (Burgdorfer et al., 1982).

In Europe the first isolation of the Lyme etiologic agent occurred in 1983, in Switzerland from *I. ricinus* ticks (Johnson et al., 1984; Barbour & Hayes, 1986), considered the main European vector. However, it was only in 1989 that the first clinical case of LD in Portugal was diagnosed by David Morais, in Évora region (Lopes de Carvalho and Núncio, 2006). Later on the same researchers confirmed a general occurrence of LD across the country, where the rural areas presented the highest risk to tick's exposure. In 1993 a new *Borrelia burgdorferi* sensu lato (*B. burgdorferi* s.l.) species, called *Borrelia lusitaniae*, was isolated for the first time from the vector (Núncio et al., 1993) and later from a human patient skin lesion in 2003 by Margarida Collares-Pereira and collaborators (Collares-Pereira et al., 2004). The LD status as notifiable disease was attributed in Portugal in 1999, by the Ministry of Health.

1.2 – *Borrelia* spirochetes

1.2.1 – Biology, Morphology and Growth

The spirochetes of *Borrelia* members are gram-negative and extracellular bacteria which have a characteristic spiral shape with a motility that permits them to move easily through high viscous media where other bacteria have a reduced or inhabited movement, or penetrate several tissues contributing for their dissemination either in the vector or in the host (Tsao, 2009). These spirochetes present a double rigid membrane with a length that varies from 10 to 30 µm and a width from 0.2 to 0.5 µm (Barbour & Hayes, 1986; Krupka et al., 2007). According to the species the flagella number range from seven to 20 and are inserted in each end of the bacteria, whose structure is a protoplasmic cylinder surrounded by a citoplasmatic membrane and the periplasm, that contains these flagella, and finally by an outer membrane associated with the underlying structures (Singh & Girschick, 2004; Karami, 2012). The flagella (periplasmatic flagella) consist mainly in two types of flagellin proteins – minor FlaA (38 KDa) and FlaB (41 KDa) (Ge et al., 1998), and are attached to each end of the cell cylinder and overlap at the cell center, giving to the spirochetes its characteristic flat wave shape and its motility (Figure 2).

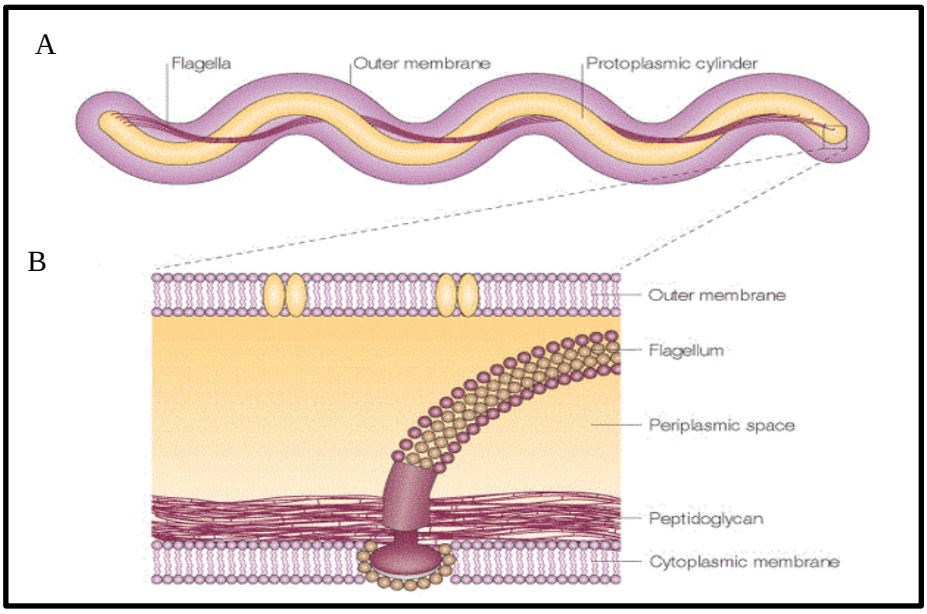


Figure 2 - Structure and morphology of *Borrelia* bacteria. A – Bacteria diagram; B – Bacteria and flagella structure. (Source: Rosa et al., 2005).

However, the spirochetes of *Borrelia* are considered pleomorphics, being able to change its morphology as a response to environmental conditions. The existence of pleomorphism among many bacterial species i*n vitro* has been known for over a century (Mattman, 2001). At the beginning of the 19th century, researchers demonstrated that spirochete species had multiple morphologies (Berndtson, 2013). Today it is well known that many Gram-negative and Gram-positive bacteria can spontaneously or by stimulation change their morphology both *in vitro* and *in vivo* (Domingue and Woody, 1997).

The cellular envelope composition is similar to the Gram negative bacteria's, however, this spirochete lacks the capacity to synthesize amino acids, nucleotides, fatty acids, and enzyme cofactors, as the genes encoding the enzymes for these pathways were presumably lost during the coevolution with its tick vector and mammalian host (Fraser et al., 1997; Gherardini et al., 2010) . Instead, *B. burgdorferi* is an accomplished importer and hunter that has at least 52 genes encoding transporters and/or binding proteins of carbohydrates, peptides, and amino acids (Saier & Paulsen, 2000). Additionally, energy is obtained from glycolysis and the fermentation of sugars to lactic acid, as the genes encoding the components necessary for the citric acid cycle and oxidative

phosphorylation are missing (Fraser et al., 1997; Gherardini et al., 2010). In addition the cell expresses several plasmid-encoded outer surface proteins (OSP) from A to F (Fraser et al., 1997; Krupka et al., 2007). These OPS's and other differentially expressed outer surface proteins apparently help the spirochete to adapt and survive in different arthropod and mammalian environments. Moreover, during the disseminated phase of infection, another surface exposed lipoprotein the VlsE, suffers extensive antigenic variation (Zhang et al., 1997). The few proteins with biosynthetic activity make the bacteria dependent on the host for much of its nutritional requirements.

Cultivation of *Borrelia* spirochetes is difficult and sometimes unsuccessful, since these bacteria require an environment low in oxygen and have very specific nutritional needs, growing in a very complex medium. Several *Borrelia* culture media were developed such as modified Kelly–Pettenkofer (MKP) medium, Barbour–Stoenner–Kelly-II (BSK-II) medium, and BSK-H, the only commercial medium (Barbour, 1984; Pollack, et al., 1993; Murray, et al., 2008). The BSK-H media is very complex containing mainly amino acids, cofactors, salts, N-acetyl-D-glucosamine and a number of variable components such as bovine serum albumin, neopeptone, yeast extract and rabbit serum. Suppliers and a batch of the referred ingredients can not only affect growth rate, but also the antigenic and morphologic characteristics of *Borreliae* (Pollack et al., 1993; Wang et al., 2004). This bacterium has the unique characteristic of growing in an iron ion-depleted environment, due to the elimination of most iron dependent metalloproteins and substituting Mn for Fe in the remaining ones (Posey & Gherardini, 2000).

The incubation temperatures range between 30 to 34º C and generation time can go from seven to 20 hours or even longer. Since there are so thin the microscopic observation is only possible with phase-contrast or dark field microscopy (Singh & Girschick, 2004). However, several staining's can be used to observe these spirochetes such as silver or immunofluorescence, using specific markers with fluorochromes (Figure 3) (Krupka et al., 2007).

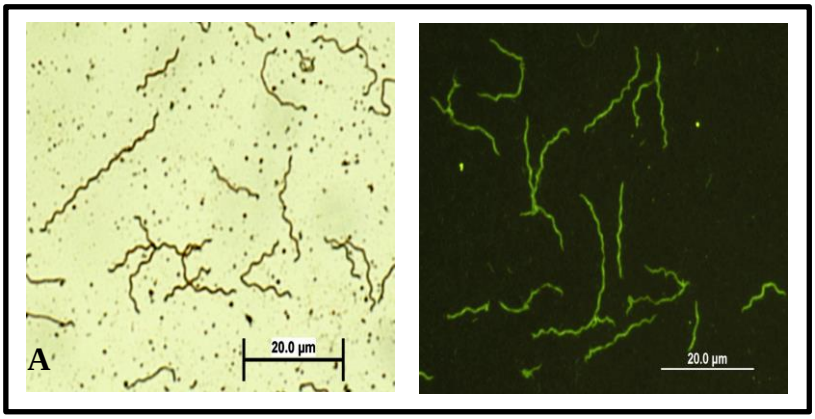


Figure 3 – Morphologic aspect of *Borrelia* spirochetes. Magnification 1000x using immersion objective. A - Warthin-Starry (silver nitrate staining); B - Immunofluorescence. (Source: Krupka et al., 2007).

The first complete sequencing of *B. burgdorferi* s.l. genome was in 1997 by Fraser and collaborators, that sequenced the strain B31 from *B. burgdorferi* sensu stricto (s.s.) (Fraser et al., 1997). Nowadays a total of eight complete genomes of *B. burgdorferi* s.l. are available in databases (Di et al., 2014). The Lyme borreliosis (LB) genome (1.5Mb), is one of the most, if not the most complex genome of any bacterium, it consists in one unique linear chromosome with about one mega base of size, and several linear (nine) and circular (21) plasmids, more than any other know bacteria. At the chromosome level high homology among species is observed, about 46-74% (Baranton et al., 1992; Postic et al., 1994; Wang et al., 1999), yet the plasmid portion varies greatly even in strains within species (Glöckner et al., 2006; Tsao, 2009).

The chromosome presents mostly genes that encoded vital functions of the spirochete, and homologs of know genes, including housekeeping genes (Rosa et al., 2005; Margos et al., 2009). By the other hand, many plasmid-encoded genes require experiments to infer their function. Some recent studies suggest that many plasmid genes encode proteins important to reproduction, infection, transmission and persistence of the bacteria in the vertebrate hosts, which will have implications on the ability of causing disease (Singh & Girschick, 2004; Glöckner et al., 2006; Karami, 2012).

1.2.2 – Classification and taxonomy

The spirochetes are one of the few major bacterial groups whose natural phylogenetic relationships are evident at the level of gross phenotypic characteristics (Wang et al., 1999). These organisms belongs to Spirochaetes phylum containing only Spirochaetes class that comprises a single order Spirochaetales. This order includes three families: Brachyspiraceae, Leptospiraceae and Spirochaeteceae (Karami, 2012), where the Spirochaeteceae family comprises *Borrelia* and *Treponema* genus, this last responsible for syphilis, a sexually transmitted disease (Wang et al., 1999; Heymann & Ellis, 2012; Karami, 2012).

The genus *Borrelia* represents a tight phylogenetic cluster, which is differentiated from other spirochetal phylogenetic groups by base signature analysis of *rrs* gene (Wang et al., 1999). More than 30 species have been identified within the genus so far (Baptista, 2006; Norris, 2012). These *Borrelia* species, based on the differences between their ecological and genetic characteristics (Barbour & Hayes, 1986), are usually categorized into three major categories, the relapsing-fever borreliae, whose members cause relapsing fever worldwide; the LB borreliae, whose members cause Lyme disease throughout the Northern Hemisphere, and the reptile-associated borreliae, whose members infect reptiles but are not known to cause disease in humans (Huang et al., 2015).

- *Relapsing Fever Borrelia (RFB) complex*

The Relapsing Fever (RF) complex includes species mostly found in soft ticks belonging to the *Argasidae* family, considered rapid-feeding ticks where their bites may go unnoticed. However, RF has also been reported in several hard ticks (ixodids), and in lice. The taxonomic position of RF spirochetes is a matter of controversy, since some studies have suggested phylogenetic clustering based on geographic differences (Old World versus New World), and other studies found RF spirochetes in hard ticks, (including *B. miyamotoi*, *B. theileri*, and *B. lonestari*), which clustered together phylogenetically suggesting this to be a separate group within the RF complex (Barbour et al., 2009; (McCoy et al., 2014; Cutler 2015; Nunes et al., 2015). There are now at least 23 validated

RF *Borrelia* species (Morais et al., 2007), although others are waiting sufficient data to achieve such status (Table 1).

Table 1 - Relapsing Fever *Borrelia* species: geographic distribution and first report.

RF Borrelia species	Geographic distribution	Reference
B. anserina	Worldwide	Sakharoff, 1891
B. baltazardii	Iran	Karimi et al., 1983
B. brasiliensis	Brazil	Davis, 1952
B. caucasica	Russia	Kandelaki, 1945
B. coriaceae	USA	Jonhson et al., 1987
B. crocidurae	West Africa	Leger, 1971
B. dugesii	Mexico	Mazzotii, 1949
B. duttonii	Africa (Central Eastern)	Novy & Knapp, 1906
B. graingeri	East Africa	Heisch, 1953
B. harveyi	East Africa	Garnham, 1947
B. hermsii	Canada, Western USA	Davis, 1942
B. hispanica	Algeria, Morocco, Portugal Spain, Tunisia	de Buen, 1926
B. japonica	Japan	Kawabata et al., 1994
B. latyschewii	Central Asia, Iran, Iraq	Sofiev, 1941
B. mazzottii	Southern USA, Mexico, Guatemala	Davis, 1956
B. merionesi	North Africa	Blanc & Maurice, 1948
B. microti	Africa, Iran	Smith & Kilborne, 1893
B. miyamotoi	Japan	Fukunaga et al., 1995
B. parkeri	Western USA	Davis, 1942
B. persica	Asia, Middles East	Dschunkowsky, 1913
B. sinica	China	Masuzawa et al., 2001
B. tanukii	Japan	Fukunaga et al., 1997
B. theileri	America, Africa, Australia, Europe	Laveran, 1903
B. tillae	Cape Verde	Zumpt & Organ 1961
B. turicatae	USA, Mexico	Brumpt, 1933
B. venezuelensis	Central & South America	Brumpt, 1921
B. recurrentis	Worldwide	Lebert, 1874

However, at least two categories of RF are known to affect humans: *i)* the louse-borne relapsing fever (also known as urban or epidemic RF) caused by *B. recurrentis*, and transmitted by the body louse *Pediculus humanus humanus*. Historically, massive outbreaks have occurred in Eurasia and Africa, especially during wartime, when people were highly parasitized with body lice (Barbour & Hayes, 1986). Currently, the disease is found only in Ethiopia and neighboring countries (Cutler 2010); *ii)* and the tick-borne relapsing fever caused by *Borreli*a species transmitted by infected soft ticks of the genus *Ornithodoros*, like for example *B. hispanica* transmitted by *O. erraticus*, and found primarily in Africa, Spain, Saudi Arabia, Asia, and certain areas of Canada and the western United States (Cutler 2015, Palma et al., 2012).

In the last years, several novel species have been described, including *B. myumii* in ticks from Tanzania (Mitani et al., 2004), *B. microti* and other species from Iran (Naddaf et al., 2015), *B. turicatae*-like in bat ticks from the United States (Schwan et al., 2009), and as of yet unnamed species from penguins in South Africa (Yabsley et al., 2012), although the species status and potential virulence of these agents for humans remain unknown.

- *Reptile-associated Borrelia (REP) complex*

The epidemiologic role of reptiles has received increasing attention, in the last years, mainly due to the international pet trade of animals originating from the wilderness (Burridge, 2011). Frequently, the imported reptiles are harboring various tick species that facilitate the introduction of nonnative tick-borne pathogens, thus significantly increasing the risk to public health (Burridge, 2011). Various emerging and/or zoonotic pathogens have been isolated and characterized from reptiles or their associated ticks (Takano et al., 2010; Pastiu et al., 2012).

The reptile-associated *Borrelia* spp (REP) have been recently discovered in reptiles and their associated hard ticks, genera *Amblyomma* and *Hyalomma* (Takano et al., 2010). *B. turcica*, a member of the REP borreliae group, was described in *Hyalomma aegyptium* ticks related with Mediterranean tortoises in Turkey (Guner, 2004). *B. turcica* has been

demonstrated to form a distinct monophyletic group showing a relationship with both RF and LB groups. Reptile-associated *Borrelia* (*Borrelia* sp.) spirochetes were also isolated from *Amblyomma geoemydae* ticks and they clustered with RFB based on another phylogenetic analysis (Takano et al., 2011). The natural cycle of Tick-Borne Relapsing Fever (TBRF) spirochetes involve a diversity of small mammals and their tick vectors (Schwan et al., 2012). They are a neglected cause of zoonotic diseases which can result in illness and even death of the hosts (Kalmar et al., 2015).

- *Borrelia burgdorferi* s.l. *complex*

Ultrastructure of *B. burgdorferi* has been the subject of several investigations in the USA and Europe, its description varies, differences in morphological criteria, such as end shape and the number of endoflagella, among isolates of diverse origins have led to the speculation that additional spirochetal species may be involve in etiology of LD (Hayes & Burgdorfer, 1993).

A major effort has been done to analyze the phenotypic and genotypic diversity of *B. burgdorferi* isolates, using Polymerase Chain Reaction (PCR) techniques, targeting 16S and 23S ribosomal DNA, *flagellin*, Outer surface protein A (*OspA*), and *Borrelia* direct repeat (*bdr*) genes, as well as intergenic spacers (Guy & Stanek, 1991; Johnson et al., 1992; Postic et al., 1994; Le Fleche et al., 1997; Rijpkerna et al., 1997; Iyer et al., 2003). It is now apparent that *B. burgdorferi* is genetically diverse, and belongs to a *B. burgdorferi* s.l. genospecies complex composed of 20 different species (Ružić-Sabljić & Cerar, 2016), (Table 2). Evolutionary changes, mutation, genetic drift, migration, and natural selection created macro evolutionary divergence of species. The prevailing data suggest that *B. burgdorferi* s.l. was once a wide-ranging species in the Northern Hemisphere that rapidly separated into the current known species (Dykhuizen & Brisson, 2010).

Table 2 – *Borrelia burgdorferi* s.l. species: geographic distribution and first report.

B. burgdorferi s.l. species	Geographic distribution	Reference
***B. burgdorferi* s.s.**	USA; Eurasia	Johnson et al., 1984
B. garinii	Eurasia	Baranton et al., 1992
B. afzelii	Eurasia	Canica et al., 1993
B. japonica	Japan	Kawabata et al., 1993
B. andersonii	USA	Marconi et al., 1995
B. tanukii	Japan	Fukunaga et al., 1996
B. turdi	Japan	Fukunaga et al., 1996
B. valaisiana	Eurasia	Wang et al., 1997
B. lusitaniae	Europe, USA	Le Fleche et al., 1997
B. bissettii	USA	Postic, 1998
B. sinica	China	Masuzawa et al.,2001
B. spielmanii	Europe	Richter et al., 2004
B. californiensis	USA	Postic et al., 2007
B. Yangtzee	China	Chu et al., 2008
B. americana	USA	Rudenko et al., 2009
B. bavariensis	Europe	Margos et al., 2009
B. carolinensis	USA	Rudenko et al., 2010
B. kurtenbachii	USA, Europe (?)	Margos et al., 2010
B. finlandensis	Europe	Casjens et al., 2011
B. chilensis	Chile	Ivanova et al., 2014

Note: the underline species have been found in/or isolated from human patients, while the others species have not been associated to humans.

1.2.3 – Epidemiology and geographic distribution of LD

Lyme disease is the world's fastest growing vector-borne zoonotic disease with cases reported in over 60 countries and endemic foci in North America, Europe, and Asia (WHO, 2013). It occurs normally in temperate areas, with the ideal climate and general conditions for the survival and maintenance of the vector life cycle involved in *B. burgdorferi* s.l. species transmission.

The geographic distribution of LD is increasing, resulting in a significant risk, for public health (Rizzoli et al., 2011). The northeastern United States is traditionally defined as the endemic global region for LD and the public health risk is highest in this area, where annually about 16 000 to 25 000 new cases occur (Figure 4). However, the CDC estimates that only 10% of LD cases are being recorded which translates into approximately 300,000 estimated cases in the United States each year, only between 1995 and 2013 Lyme cases has increased about 130% from 11.700 to 27.200 (CDC).

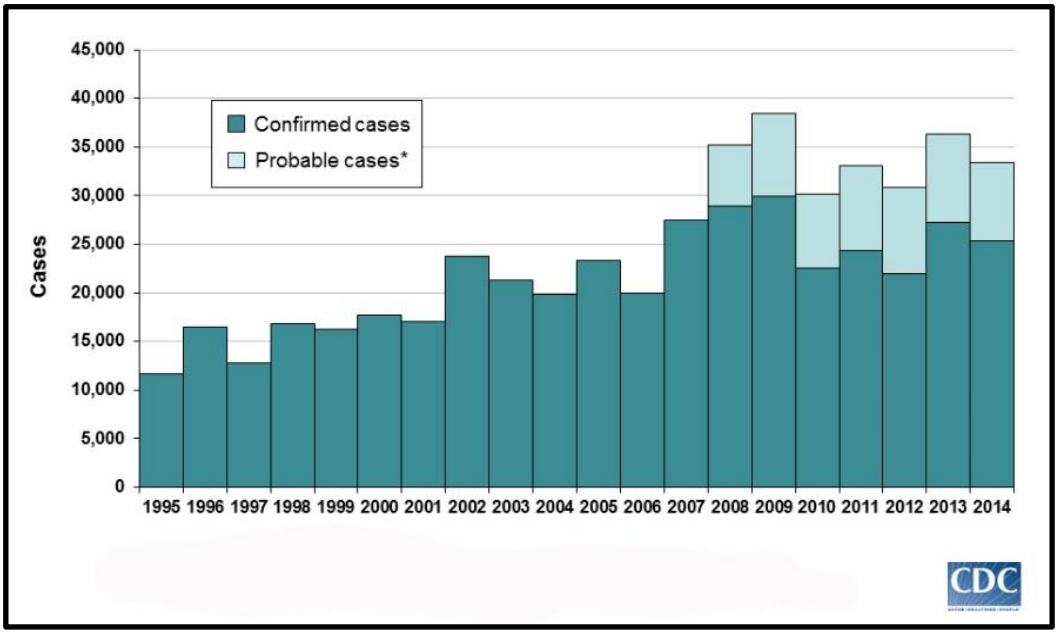


Figure 4 - Graphical representation of Lyme disease confirmed and probable annual cases in USA, from 1995 to 2014. (Source: http://www.cdc.gov/lyme/stats/graphs.html).

A majority (95 %) have been concentrated in the northeast, yet cases have been reported in every state and in many countries around the world and their geospatial analysis reveals that LD has extended well beyond traditionally defined endemic areas (CDC, 2014; Diuk-Wasser et al., 2012). Lyme disease rates have been increasing exponentially at the global scale while in the United States it has become the main human vector-borne disease (Abbott, 2006; Piesman & Eisen, 2008). This increase is due to a variety of influences like climate change resulting in the expansion of the *Ixodes* tick territory including expansion to higher elevations, changes in small mammals and deer populations, changes

in deforestation and development, and improved reporting and aware (Lindgren et al., 2000; Qiu et al., 2008; Gray et al., 2009; Gilbert et al., 2014).

LD can also be found throughout Europe, Russia, and Asia (Figure 5). The highest reported frequencies of the disease are in central Europe and Scandinavia, particularly in Germany, Austria, Slovenia, the Baltic coastline of Sweden, and some Estonian and Finnish islands, where reported incidence rates are greater than 100 cases per 100,000 inhabitants (Lindgren & Jaenson, 2006; Rizzoli et al., 2011). Seroprevalence studies conducted in individual countries in recent years, including Germany, Denmark, and Sweden, have typically found positive rates less than 10%, although rates as high as 47.9% were recorded in high-risk groups (e.g., farmers and forestry workers) in Poland (Lindgren & Jaenson, 2006; Dessau et al., 2010; Dehnert et al., 2012).

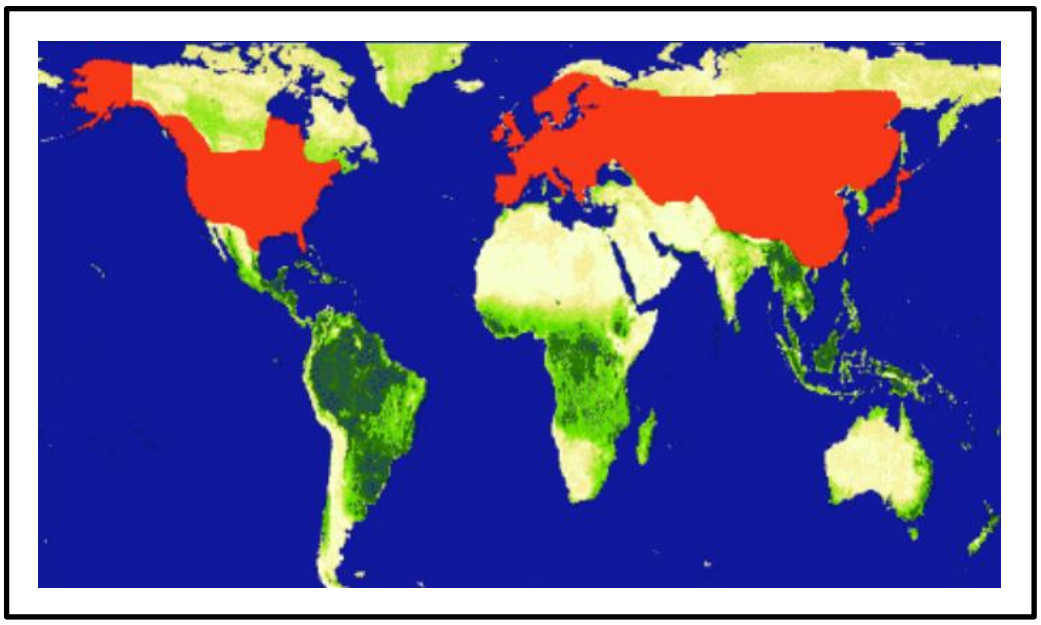


Figure 5 - World distribution of Lyme disease. In red are indicated the "hot zones". (Source: World Health Organization).

In Europe several species of *B. burgdorferi* s.l. complex have been identified being LD mostly associated to 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 species of *B. burgdorferi* s.l. have already been associated to human cases, like *B. bissettii*, *B. valaisiana*, *B. lusitaniae*, and *B. spielmanii* (Picken et al., 1996; Rijpkerna et al., 1997;

Collares-Pereira et al., 2004; Fingerle et al., 2008). In contrast, in the USA, despite the presence of several other genospecies (*B. americana*, *B. andersonii*, *B. californiensis*, *B. carolinensis*, *B. bissettii* and *B. kurtenbachii*), only *B. burgdorferi* s.s is recognized as causing LD (Murray & Shapiro, 2010). Moreover, a new *B. burgdorferi* s.l. genospecies (candidatus *Borrelia mayonii*) was recently identified among patients and in *I. scapularis* ticks from the upper Midwestern USA (Pritt et al., 2016).

The distribution and prevalence *B. burgdorferi* s.l. species varies on a local and regional scale, both temporally and spatially (Rauter & Hartung 2005; Estrada-Peña et al., 2011), with a higher biodiversity of species between 4º W and 20º E coordinates, where there is a higher prevalence of ticks infected with *Borrelia* (Estrada-Peña et al., 2011). The species *B. burgdorferi* s.s. is reported more frequently in east, while *B. afzelii* is more common in the North of Europe, *B. valaisiana* is mainly present in low temperature regions with undergrowth vegetation like Scandinavian coast, Scotland or Alpine regions, and *B. lusitaniae* and *B. garinii* are predominantly found in the Mediterranean region, southeast and west (Figure 6) (Franke et al., 2013).

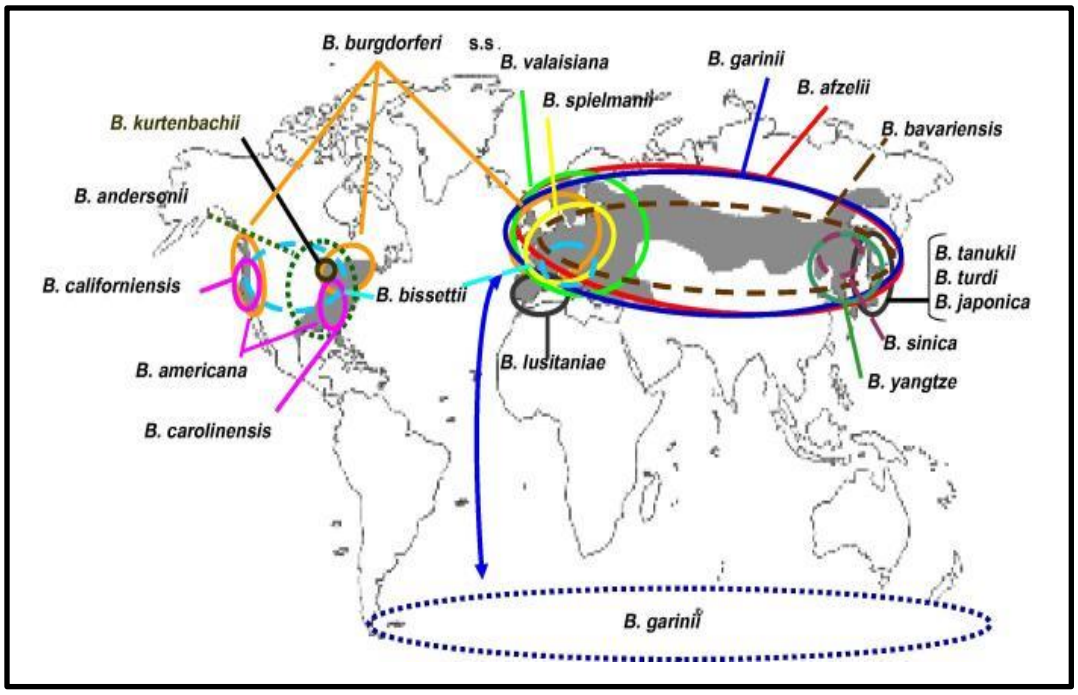


Figure 6 – Global distribution of the Lyme disease species. The shaded areas show the distribution of tick vectors. Seven species are found in North America, eight species in Europe, and eight species in Asia. (Source: Margos et al., 2011).

According to Stanek and Reiter (2011), some investigators recognize that the multiplicity of *B. burgdorferi* s.l. species existents in Europe may indicate these spirochetes as responsible for Lyme while emergency disease in this continent. However, other studies showed the existence of a more close relationship between the European *Borrelia* species than the ones from North America, suggesting that LD was introduced in Europe from the American continent (Stanek & Reiter, 2011).

The heterogeneity of *B. burgdorferi* s.l. species in Asia is similar to those in Europe, six pathogenic species and five 'potential' pathogenic have been identified in Asian continent. Almost all European species can be found in Central and Eastern Asia, yet *B. lusitaniae* is mainly found in western Asia ticks (Franke et al., 2013). *B. burgdorferi* s.s. seems absent in most Asiatic regions, being rarely reported in Thailand and South China (Franke et al., 2013), while *B. garinii* and *B. afzelii* are the main species involved in LD cases in this continent (Schotthoefer & Frost, 2015).

In North Africa several *Borrelia* species can be found particularly in the most humid and temperate regions like Tunisia, Egypt, Morocco, and Algeria. *B. lusitaniae* is the predominant species in these regions, although *B. garinii*, *B. burgdorferi* s.s. and *B. valaisiana* have been occasionally reported (Franke et al., 2013). *B. lusitaniae* isolates from North Africa suggests that they may be originate from a Portuguese clone (Stanek et al., 2012).

In Australia there are no concrete evidences of LD existence, since the bacteria as never been isolated from the tick vector, however, it has been associated to *Ixodes holocyclus* or *I. cornuatus* (Mayne et al., 2014). The majority of reported human cases are based in serologic tests, yet in 2011 DNA from *B. burgdorferi* s.l. spirochetes was detected in eight Australian patients by PCR analysis, whereas only one of the patients had left the country (Franke et al., 2013).

More recently in South America, particularly in Brazil LD, known as Brazilian Lyme-like disease or Baggio-Yoshinari syndrome, has been poorly studied (Yoshinari et al., 2010), its epidemiology and the prevalent genospecies are still not well defined (Dantas-Torres et al., 2008). However, some cases of this disease have been reported in humans and animals by serologic methods and/or by clinical symptoms in the northern (Amazonas

and Tocantins States) (Abel et al., 2000; Carranza-Tamayo et al., 2012), midwestern (Mato Grosso do Sul State) (Naka et al., 2008; Carranza-Tamayo et al., 2012), southeastern (Espírito Santo, Rio de Janeiro and São Paulo States) (Azulay et al., 1991; Yoshinari et al., 2003; Passos et al., 2009) and southern (Parana State) (Gonçalves et al., 2014; 2015) regions of Brazil. Most of these cases were detected in inhabitants of rural areas, where due to the close proximity of humans to the animal population often parasitized by ticks, results in a high incidence of this zoonosis.

Nonetheless, some recent studies have molecularly identified *Borrelia* DNA in three peripheral blood samples collected from humans with clinical symptoms of borreliosis and report of tick exposure (Mantovani et al., 2012), and also in two *Dermacentor nitens* tick species (Gonçalves et al., 2014). More recently, *B. garinii* and *B. burgdorferi* s.s. were reported for the first time in residents of rural areas, who were directly or indirectly exposed to wild and/or domestic animals and ticks in the northern region of Parana State, confirming the presence of these genospecies in Brazil (Gonçalves et al., 2015).

- *Lyme disease in Portugal*

In Portugal the first human case of LD was identified in 1989 by David Morais and collaborators in Évora region. In this work the authors suggested, either new vectors could be implicated in the transmission of *B. burgdorferi* s.l., since *I. ricinus* species was considered uncommon in that region, or the potential existence of a new *Borrelia* strain in the country. Subsequent studies in the same Portuguese region confirmed the presence of more seropositive cases, some of them with confirmed clinical signs of LD (Filipe et al., 1990, Núncio et al. 1992; David de Morais & Henriques, 1999). Ten years later this zoonosis was considered a notifiable disease to the Portuguese Health Authorities (Portaria 1071/98, A69.2).

The first isolated strains of *B. burgdorferi* s.l. were obtain from ticks collected in the South of Portugal, where a new species was identified firstly as PoTi B1, and later designated as *B. lusitaniae* (Núncio et al., 1993). Further studies confirmed the presence of others species of *B. burgdorferi* s.l. in ticks (*B. afzelii, B. garinii, B. valaisiana and B. burgdorferi* s.s.) with different prevalence rates from 11.9% in several regions, to 31.2%

in Madeira island and 34.7% in Grândola region. Although in every reported studies done in Portugal until 2006, *B. lusitaniae* was always the most prevalent species (Lopes de Carvalho & Núncio, 2006).

A study developed between 1990 and 2004 by Investigators of the Centre for Vectors and Infectious Diseases (CEVDI) at Instituto Nacional de Saúde Dr. Ricardo Jorge (National Institute of Health, INSA), located in Setubal district, showed that in these 15 years the number of patients diagnosed with LD, according to EUCALB criteria, has increased. However, the notification of LD does not follow this evolution. The Figure 7 shows the difference between the notifiable cases (represented in black) and the confirmed cases (represented in blue), based on the mentioned institute during the same period, where is clear that LD is underreported in the country.

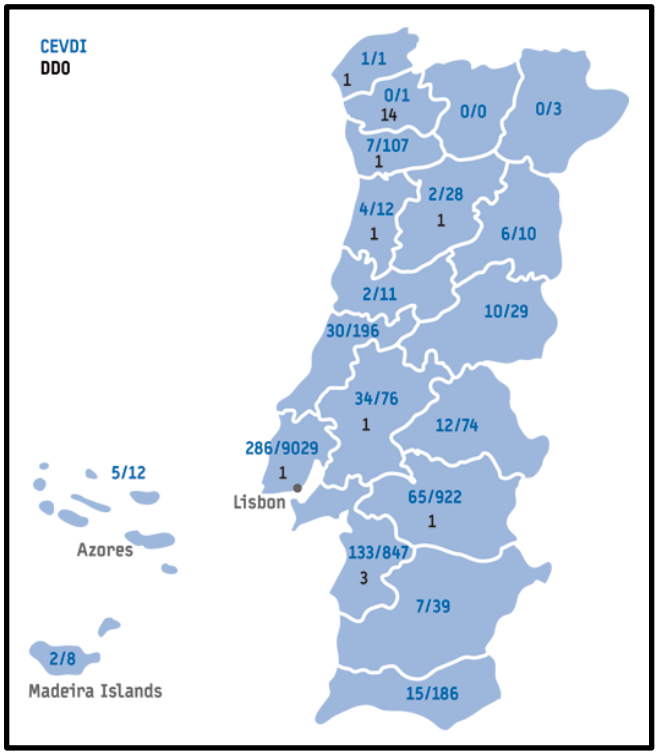


Figure 7 - Geographical distribution of LD cases (in blue) diagnosed at CEVDI and the notifiable cases (in black), by district between 1990 and 2004. (Source: Lopes de Carvalho & Núncio, 2006).

Currently in Portugal, the LD incidence (based on notifiable cases) is about 0.04 cases/100 000 population. However, if the global laboratorial data were included the incidence would increase to 0.4 cases/100 000 population, about 10 times more cases (Lopes de Carvalho & Núncio, 2006). Data from Direção Geral de Saúde (DGS), regarding the notifiable cases reported between 2009 and 2012, are represented in Figure 8, where only a total of 20 LD human cases were notifiable by the Administração Regional de Saúde (ARS).

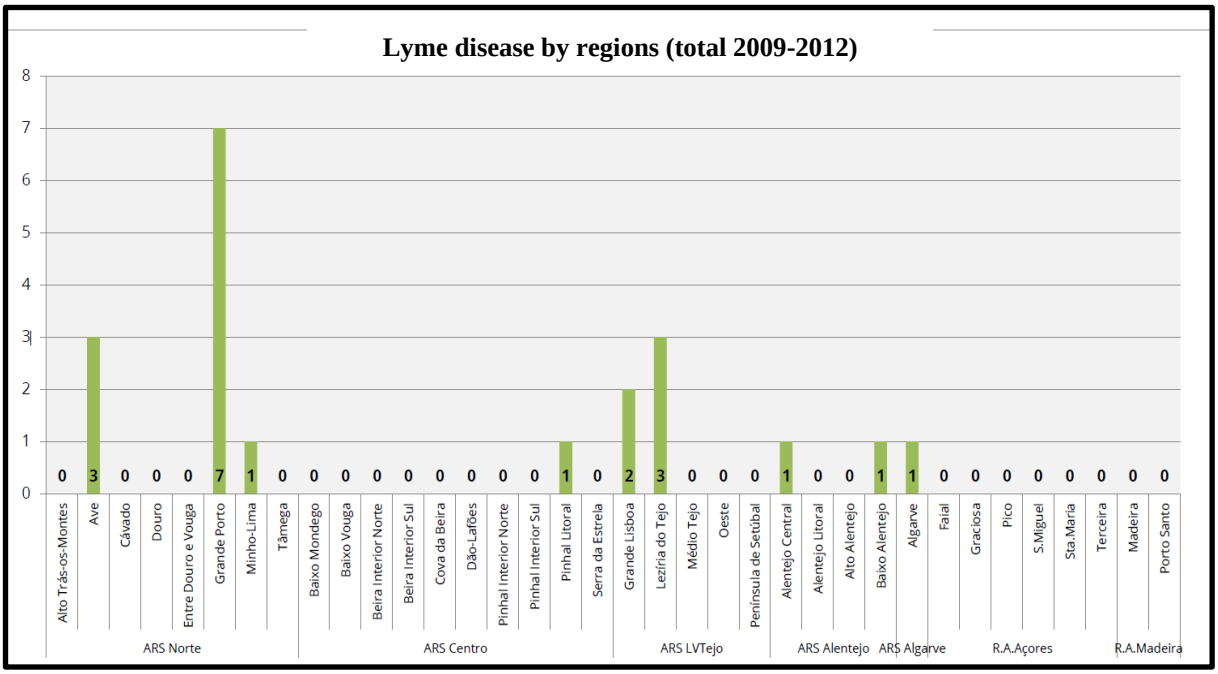


Figure 8 – Notifiable cases of Lyme disease by ARS (Administração Regional de Saúde), between 2009 and 2012. (Source: Direcção Geral de Saúde (DGS), report).

1.3 – The tick vector

1.3.1 – Classification and taxonomy

Ticks are invertebrates classified as small Arachnids in the phylum Arthropoda. Along with, they constitute the subclass Acari. These Ixodids are obligate hematophagous that parasite a wide variety of vertebrate hosts like mammals, birds, and reptiles around the world (Sonenshine, 1992).

According to Silva and collaborators (2006), there are about 850 tick species, distributed in three families: Nuttalliellidae, Argasidae and Ixodidae (Figure 9). The Nuttalliellidae family have a single species, and is geographically limited to Southern Africa (Oliver, 1989); Silva et al., 2006). The Argasidae family includes about 170 species, are also designated by soft-body ticks, since they don't have dorsal Scutum, presenting a flexible cuticle (Sonenshine, 1992). Regarding the Ixodidae family, it comprehends the highest number of species, about 650, also known as hard-body ticks, due to their dorsal sclerotized plate. Ticks of this family are considered of great medical and veterinary importance due to the number of species involved in the transmission of several pathogens to humans and other vertebrate animals, with great relevance in zoonotic diseases such as Lyme borreliosis (Silva et al., 2006).

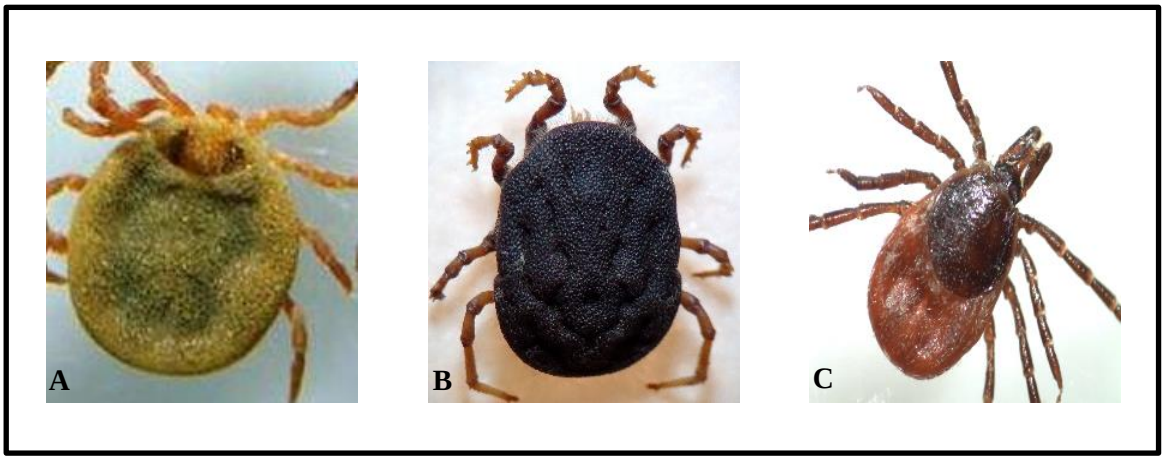


Figure 9 - Examples of tick species from each family. A – *Nuttalliela namaqua* from Nattalliellidae family; B – *Ornithodoros erraticus* from Argasidae family; C – *Ixodes ricinus* from Ixodidae family. (Source: A and B Photos from google images; C – original photo by Mónica Nunes).

Ticks are only second to mosquitoes as worldwide vectors of agents causing human diseases, but the most important vectors of animal pathogens (de la Fuente et al., 2008). The success as potential vectors is due to their unique biological characteristics detailed in Table 3.

Table 3 - More important biological characteristics of ticks. (Source: Adapted from Silva et al., 2006).

	Biological characteristics
Ticks	Obligated hematophagous;
	Slow feeding allowing an wide period of interaction with the host;
	Life cycles mono, bi, and three-phase depending if they feed on one, two or three vertebrate hosts;
	All phases ingest high volumes of blood;
	The digestion is gradual and intracellular, occurring in the lumen without enzymes;
	Transstadial and transovarial transmission of pathogenic agents;
	Developed sensorial system that allows to detect the presence of hosts by the CO_2 emissions, of lactic acid, ammonium and other odors;
	Diapause;
	Wide longevity and high fertility rates;
	Worldwide distribution, present in almost all terrestrial habitats;
	Low number of natural predators.

Generally, arthropods can act as mechanical or biological vectors. In the first case, the pathogenic agent does not multiply in the vector, and is transmitted mechanically from one host to another. The pathogenic agent can adapt to arthropod midgut conditions, until it is transmitted to the vertebrate host. In the case that the arthropod acts as a biological vector, the pathogenic agent has the ability to invade its tissues before being transmitted to the host, this situation is the most frequent in tick-borne pathogens transmission (Silva et al., 2006).

Ticks are normally zoophilic (feeding on animals), and some species have been found parasitizing humans, considered as accidental hosts. In order for ticks to transmit effectively a pathogenic agent, they must have the ability in space and time to transmit the agent – vectorial capacity; and competence - the intrinsic capacity of maintaining the agent and biologically transmit it during the blood meal (Silva et al., 2006).

1.3.2 – Hard-ticks morphology

The ixodids are characterized by the presence of a tough sclerotized plate on the dorsal body surface, the scutum, covering the entire dorsal body surface in males (sometimes named conscutum), and limited to the anterior dorsal surface, covering approximately one third of the dorsal body region in unfed females, nymphs and larvae. The folded cuticle posterior to the scutum constitutes the alloscutum. Both scutum and alloscutum are covered with numerous small setae. Sexual dimorphism is apparent only in the adult stage. The scutum is the site of attachment of various dorso-ventral body muscles, cheliceral retractor muscles, and many other muscle groups in these ticks. Eyes, if present, are located on the lateral margins of the scutum (Figure 10) (Estrada-Peña et al., 2004).

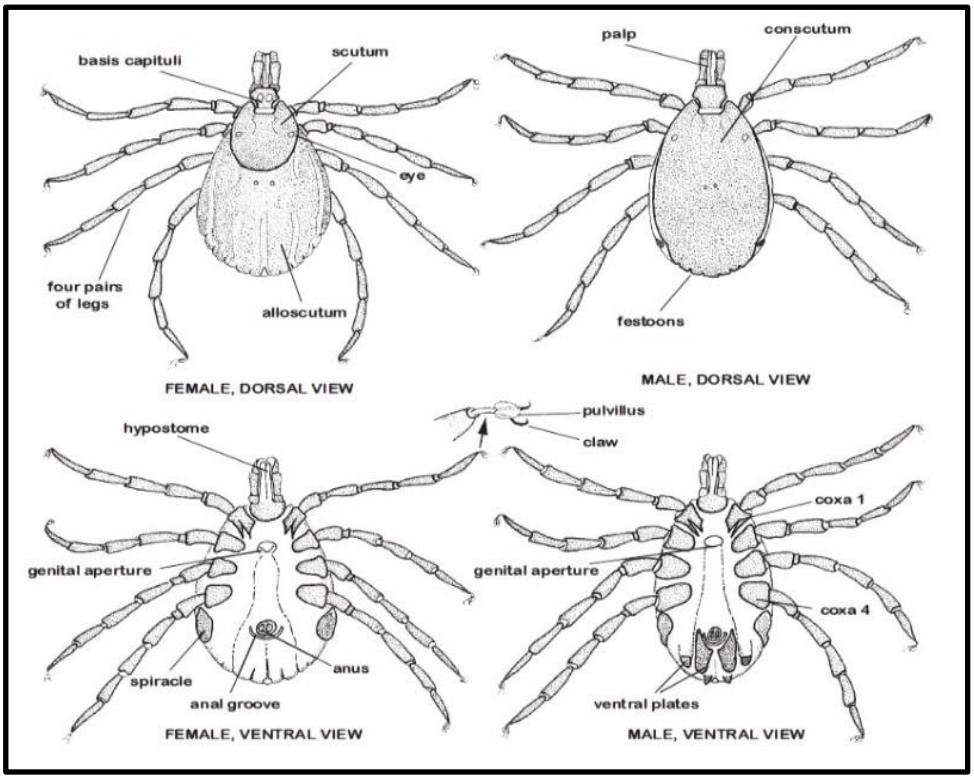


Figure 10 - External morphology of ixodid ticks from ventral and dorsal side.
(Source: Estrada-Peña et al., 2004).

Ventrally, nymphs and adults bear a pair of spiracular plates (= stigmata) located immediately posterior to the fourth coxae, with the spiracle, a single opening, within each plate.

Sexual differentiation is not seen in immature tick stages. However, males and females have a genital aperture, the gonoporum, on the ventral side of the idiosoma, between the coxae of the second pair of legs. In *Ixodes* genus the anus is also located on the ventral side, near the posterior margin of the body, behind the fourth pair of legs, and is associated with being surrounded by the anal groove. This groove may be absent or located anterior or posterior to the anus, depending of the genera (Estrada-Peña et al., 2004; Márquez-Jiménez et al., 2005). Only *Ixodes* genus presents an anterior anal groove (Figure 11).

Figure 11 - Ventral side of *Ixodes ricinus* female showing the anterior anal groove. (Original photo by Mónica Nunes).

1.3.3 – Hard-ticks species in Portugal

In Portugal the ecological, climatic and environmental conditions have been favorable to the development and maintenance of several hard-ticks, existing about 21 species from five different genera in the country (Table 4).

Table 4 - Hard-tick genera and respective species present in Portugal.

Tick genera	Ticks species	Reference	Specimen example (original photos by Mónica Nunes)
Dermacentor **(n=2)**	*marginatus*	Sulzer, 1776	
	reticulatus	Fabricius, 1794	
Haemaphysalis ***(n=3)***	*hispanica*	Gil Collado, 1938	
	inermis	Birula, 1895	
	punctata	Canestrini & Fanzago, 1878	
Hyalomma **(n=2)**	*lusitanicum*	Koch, 1844	
	marginatum	Koch, 1844	
Ixodes **(n=10)**	*acuminatus*	Neumann, 1901	  
	arboricola	Schulze & Schlottke, 1930	
	bivari	Dias, 1990	
	canisuga	Johnston, 1849	
	frontalis	Panzer, 1798	
	hexagonus	Leach, 1815	
	ricinus	Linnaeus, 1758	
	simplex	Neumann, 1906	
	ventalloi	Gil Collado, 1936	
	vespertilionis	Koch, 1844	
Rhipicephalus **(n=4)**	*boophilus annulatus*	Say, 1821	
	bursa	Canestrini & Fanzago, 1878	
	pusillus	Gil Collado, 1938	
	sanguineus	Latreille, 1806	

Regarding *Rhipicephalus* genera, the species *R. turanicus* had been previously reported in mainland Portugal (Papadopoulos et al., 1992; Dias et al., 1994; Caeiro, 1999; Estrada-Peña et al., 2004; Santos-Silva et al. 2006) , although, its presence on the Mediterranean

area has been questioned. According to the opinion of Walker and collaborators (2000), about the genus *Rhipicephalus*, and together with the recent molecular data analysis from Santos-Silva and collaborators, based on three mitochondrial genes [12S rDNA, cytochrome c oxidase subunit II (COXII) and the control region or d-loop (DL)] and one nuclear gene (28S rDNA), the ticks commonly called *R. turanicus* in Portugal by morphological analysis are genetically indistinguishable from *R. sanguineus*, pointing towards to the occurrence of a single species in Portugal, *R. sanguineus*, characterized by a high level of morphological polymorphism (Santos-Silva et al., 2011).

These different tick species can transmit a large variety of pathogenic agents able of causing disease in humans, with an emergent risk in Portugal (Table 5).

Table 5 - Etiologic agents transmitted by Ixodids present, or at emerging risk, in Portugal. (Source: adapted from Núncio & Alves, 2014).

Pathogenic agent	Disease	Ixodid species
Anaplasma phagocytophilum	Human anaplasmosis	*Ixodes ricinus, I. ventalloi*
Babesia divergens	Babesiosis	*Ixodes* spp.
***Borrelia burgdorferi* s.l.**	Lyme borreliosis	*Ixodes ricinus*
Coxiella burnetii	Q Fever	Several species
Francisella tularensis	Tularemia	Several including *Ixodes ricinus* and *Dermacentor reticulatus*
Rickettsia aeschlimannii	without nomination	*Hyalomma marginatum*
R. conorii	Mediterranean Spotted Fever	*Rhipicephalus sanguineus*
R. helvetica	without nomination	*Ixodes ricinus*
R. massiliae	without nomination	*Rhipicephalus sanguineus*
R. monacensis	without nomination	*Ixodes ricinus*
R. sibirica mongolotimonae	LAR*	*Hyalomma sp., Rhipicephalus pusillus*
R. slovaca	TIBOLA**	*Dermacentor marginatus, D. reticulatus*
Crimean-Congo hemorrhagic fever virus	Hemorrhagic fever	*Hyalomma marginatum, Haemaphysalis punctata, Ixodes ricinus, Dermacentor* spp. *Rhipicephalus* spp.
Eyach virus	without nomination	*Ixodes ricinus, Ixodes ventalloi*
Tick-Borne Encephalitis virus	Encephalitis	*Ixodes ricinus, Haemaphysalis punctata*

* LAR - Lymphangitis-associated rickettsiosis; ** TIBOLA - Tick-borne lymphadenopathy

Lyme disease agents have been isolated and identified from different hard-tick genera, although some ticks constitute a greater risk of transmitting these agents to humans than others (Silva et al., 2006; Franke et al., 2013). These spirochetes are carried mainly by ticks belonging to *Ixodes* genus from the Ixodidae family (Parola & Raoult, 2001), currently comprehending four predominant species, *I. scapularis*, *I. pacificus*, *I. ricinus*, and *I. persulcatus* (Piesman & Gern, 2004; Stanek et al., 2012). Many, if not all, species of this complex are important vectors of other pathogens that cause human and livestock diseases, including tick-borne encephalitis, anaplasmosis and babesiosis. Therefore, throughout this study, emphasis will be given to tick's representative of *Ixodes* genus, classified as competent vectors, and more directly involved in *B. burgdorferi* s.l. species transmission.

1.3.4 – Geographic distribution of *Ixodes* vector

There are four predominant species of *Ixodes* ticks associated to spirochetes transmission to humans, including *I. scapularis* in the eastern United States and Canada, *I. pacificus* in the western USA, *I. ricinus* in Europe and Asia, and *I. persulcatus* in Asia (Figure 12) (Piesman & Gern, 2004; Stanek et al., 2012).

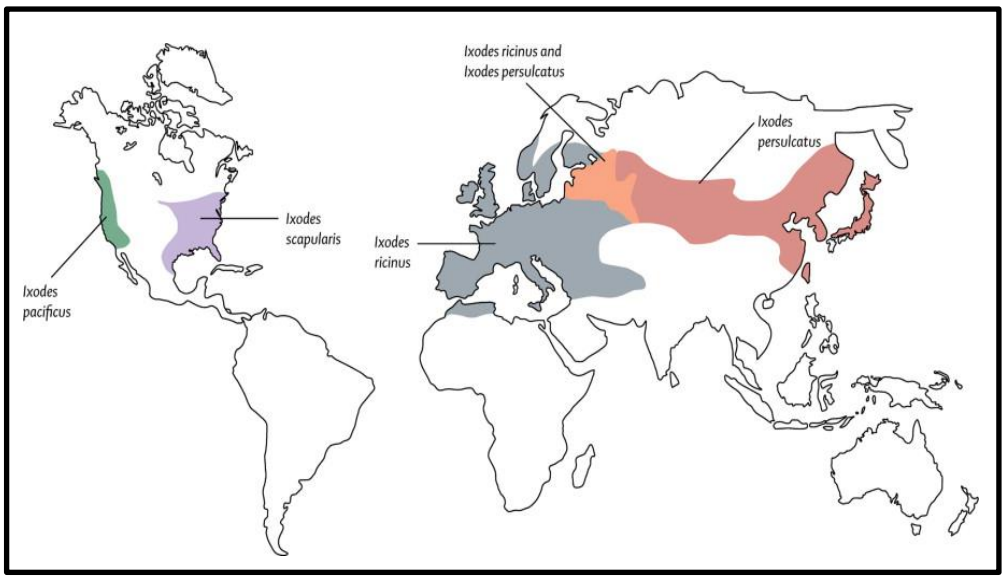


Figure 12 – Geographical distribution of *Ixodes* species, vectors of Lyme disease agents. (Source: Stanek et al., 2012).

The European tick, *I. ricinus* species, also known as sheep tick or Castor bean tick, presents a wide geographic distribution across Europe, due to abiotic and biotic factors such as specific microclimate, biotopes, and host dynamics (Movila et al., 2012), and transmits an even greater array of pathogens than its "sister" in North America, the species *I. scapularis* (Lindgren & Jaenson, 2006; Gray et al., 2009). *I. ricinus* tick has a high affinity for humans, making it the most important bridging vector in Europe (Guiguen & Degeilh, 2001; Parola & Raoult, 2001). In the last decades the global warming influenced the distribution and abundance of this vector, being present from the Faroe Islands in the west (Jaenson & Jensen, 2007) to the European section of the Russian Federation in the east (Korenberg et al., 2002), and from North Africa (Zhioua et al., 1999) to the Northern Scandinavia (Lindgren et al., 2000), (Figure 13).

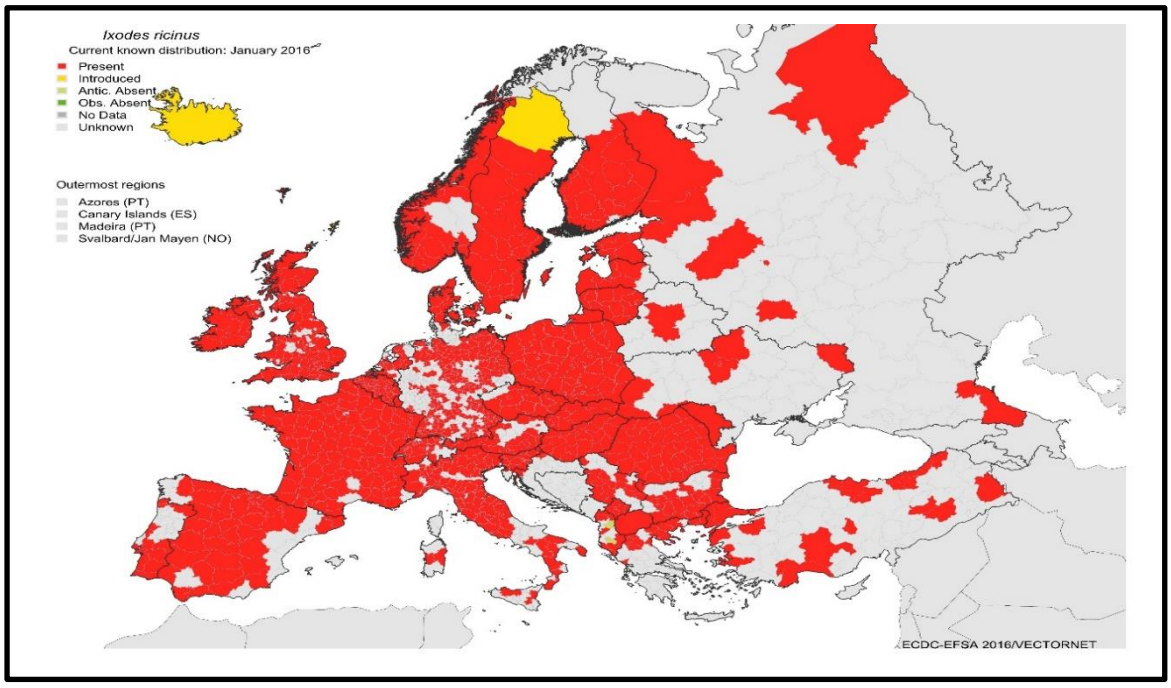


Figure 13 - Geographical distribution of *Ixodes ricinus* in Europe. (Source: ECDC, 2016).

This tick has the particularity of questing to the tip of low vegetation to meet its hosts. During this questing, ticks often have to face desiccating conditions, quitting their questing place and moving to the liter/mat layer where they regain lost body water (Randolph & Storey, 1999; Gern et al., 2008). *Ixodes ricinus* moves preferentially when desiccation risk is the lowest in nature, at sundown (Gern et al., 2008). If high desiccating conditions are lasting too long, tick mortality is increased resulting in questing tick

population decrease (Perret et al., 2004). This tick is sensitive to climatic conditions, requiring a relative humidity of at least 80% to survive during its off-host periods, being therefore restricted to areas of moderate to high rainfall with vegetation that retains a high humidity (Medlock et al., 2013). Recently, this tick species has expand in terms of altitude and latitude, mainly due to climatic changes, leading to the colonization of new habits and modifications in the seasonality patterns (Santos-Silva et al., 2011).

In Portugal *I. ricinus* species can be found across the country, being most predominant in areas of deciduous woodland and mixed forest with mild temperatures, where relative humidity levels are high (Silva et al., 2006). In unfavorable conditions (absence of vegetation and high temperature), the vitality of each stage can be compromised, leading them to find suitable refuges to their survival, and to use survival strategies such as diapause (Schwarz et al., 2012; Stanek et al., 2012).

1.3.5 – Life cycle of *Ixodes ricinus*

The tick *I. ricinus* is a triphasic (three hosts), exophilic (finds its host in an open environment) and telotrophic species (the immature stages feed in different hosts, including those where the adults feed), that can take about three years to complete its life cycle. This tick, like all hard-body ticks, has three postembryonic development stages - larva, nymph, and adult (Figure 14).

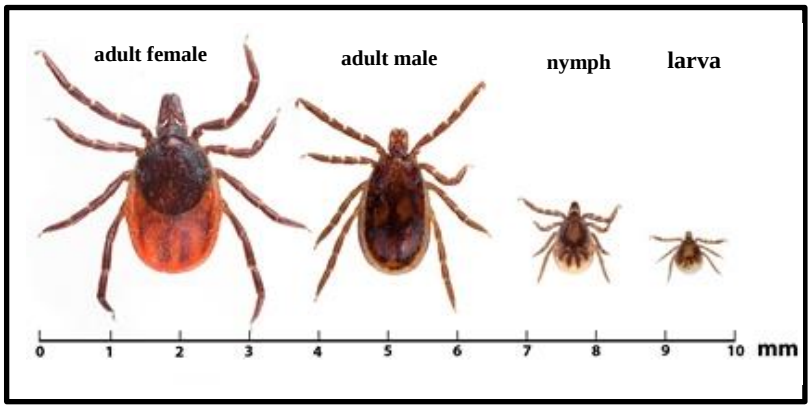


Figure 14 - *Ixodes ricinus* life stages.
(Source: adapted from http://www.alleskatten.nl/gezondheid/pathologie-en-farmacologie/).

Their activity occurs during all year, however, it presents a specific seasonality, being adults more active between autumn (October) and spring (March), while larvae and nymphs are more active, in the host and also in the vegetation, between spring and summer (April to July). The duration of the life cycle depends of important factors as climate and host availability (Estrada-Peña et al., 2004; Stanek et al., 2012; Handeland et al., 2013).

The immature stages (larva and nymph) remain mainly in low vegetation, where larva feed primarily on small mammals (rodents and rabbits), and nymphs are found in hosts of medium size like birds and reptile, the adults feed on a variety of large animals (Figure 15). With the exception of the adult male that takes small blood meals and do not engorge, each life stage requires a blood meal from a vertebrate host. Both genders can also be found in high vegetation, where they expect potential hosts. Only one blood meal is made in each evolutional stage of the tick (Mannelli et al., 2012; Movila et al., 2012).

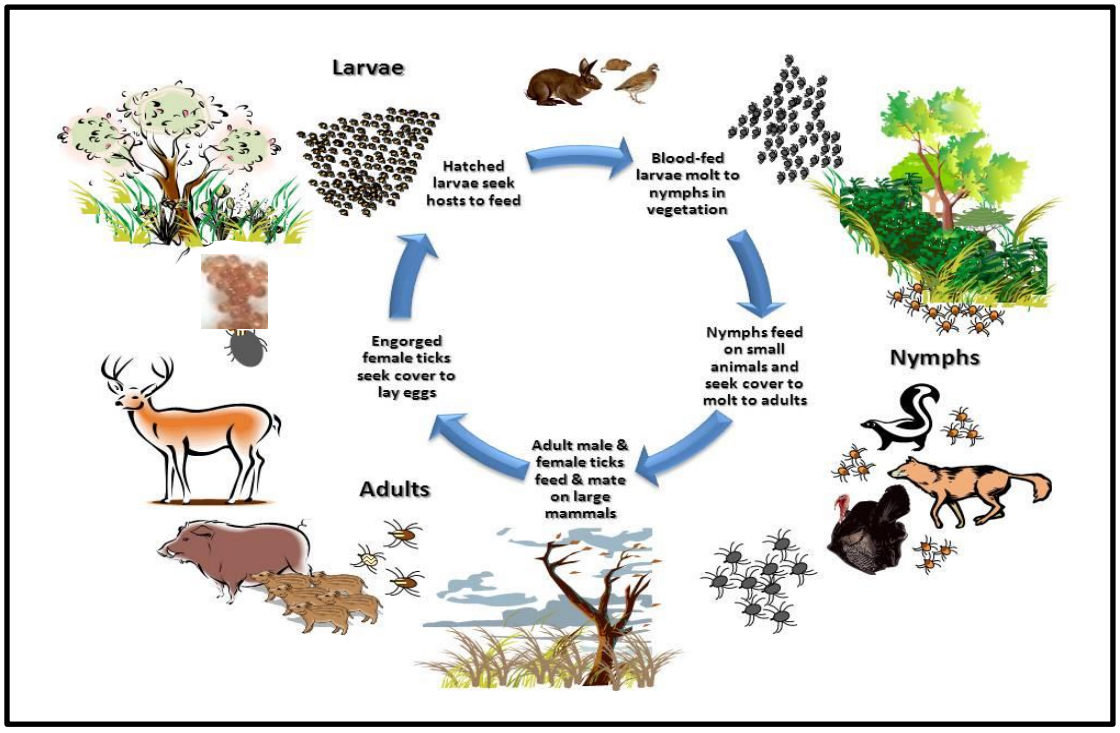


Figure 15 - Life cycle of *Ixodes ricinus* ticks. (Source: adapted from http://tickapp.tamu.edu/tickbiology.php)

All stages of *I. ricinus* species use the same technique to hold on to the host, they normally climb to the top of the vegetation and when the host passes the tick grabs to the fur or

skin through its questing legs, and then bites using its specialized mouthparts. After it has finished its blood meal, which can last several days (3-4 days to larvae, 4-8 days to nymphs and 5-20 days to adult females), contributing to their geographical spread along with the movement of the host (Wilske, 2005), it loosen up from the host to the soil, and molt to the next stage. The life cycle of *I. ricinus* ends with the mating, where the female tick after fully engorged produces eggs and deposited them in the soil (oviposition). After the posture of the eggs (approximately 2000), the female dies (Estrada-Peña et al., 2004; Stanek et al., 2012; Medlock et al., 2013).

1.3.6 – Transmission and Pathogenesis

Borrelia burgdorferi s.l. spirochetes can be transmitted to the tick by three possible ways: *i)* through the blood meal in an infected host (the most common way); *ii)* by transovarial transmission (TOT) and/or *iii)* by transstadial transmission (Figure 16).

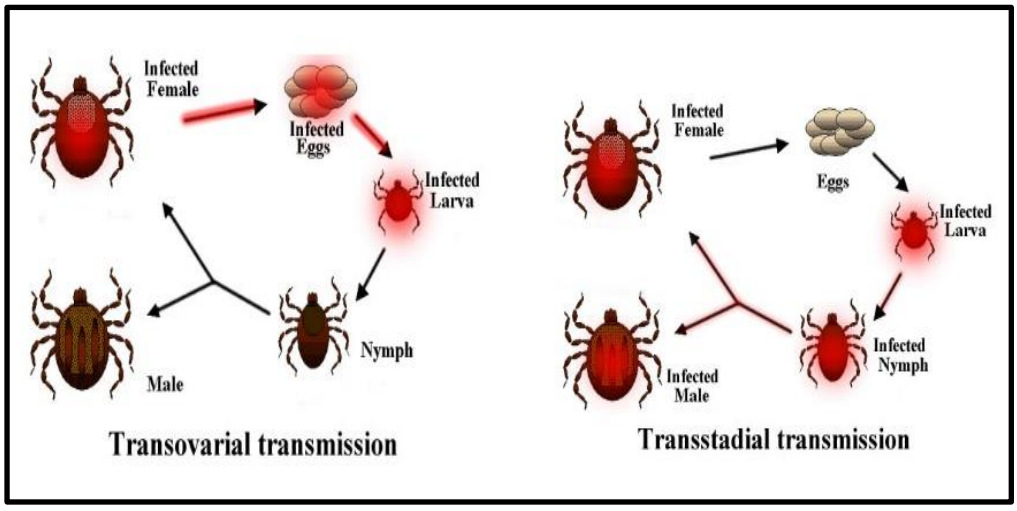


Figure 16 – Schematic representation of transovarial and transstadial transmission of pathogenic agents in *Ixodes* ticks. (Source: https://en.wikipedia.org/).

The transovarial transmission of spirochetes is rare, however, this hypothesis has been explored by many tick-borne pathogens for maintenance in natural environment and reported to occur in both ixodid and argasid ticks (Rollend et al., 2013). Although studies carried out both in the USA and Europe have shown *B. burgdorferi* s.l. in *I. scapularis* and *I. ricinus* larvae (Rijpkema et al., 1994; Hubálek & Halouzka, 1998), this seems to

occur at a very low rate. For this reason, TOT does not seem to play any significant role for the natural maintenance of *B. burgdorferi* s.l. and a consequent minimal contribution to the dynamics of infection in adult ticks of the next generation is expected (Nefedova et al., 2004).

Ticks transmit the spirochetes by cutaneous inoculation of infected saliva. After the tick attaches, the spirochetes disseminate from the skin, to other tissues, organs or systems, through the blood flow. If the tick has been attached less than 24h, the risk of infection is low (Piesman, 1993), since the transmission of *Borrelia* is more efficient as greater is fixation time of the tick to the host, being necessary an attachment of 48-72h for a successful transmission of the spirochete. However, several authors verified that the attachment time to the host depends on the *Borrelia* species and also on the vector (Stanek et al., 2012). For example, the spirochete of *B. burgdorferi* s.s. is not transmitted before 48h of attachment, while *B. afzelii* can be transmitted in less than 24h. Also, *I. ricinus* can transmitted the spirochetes faster than *I. scapularis* and *I. pacificus* (Marques, 2010; Wood & Lafferty, 2013).

During the attachment, ticks injects a complex mixture of bioactive chemicals into the host, like histamine binders and cytokine inhibitors to mediate the host response, complement inhibitors to suppress the host immune response, and anticoagulants to facilitate the blood meal (Müller-Doblies & Wikel, 2005). This results in a painless “bite” and usually prevents an inflammatory response. Spirochetes disseminate, along with the blood meal, from the infected host to the tick, and colonize the midgut. They remain in the midgut multiplying until the next blood meal, when a fraction of the spirochetes from the midgut invade the salivary glands. While spirochetes are in the midgut, they express high levels of OspA, since its presence is a requirement for survival in the tick by facilitating adhesion of the spirochete to the midgut wall (Pal et al., 2000), biding to a receptor TROSPA (tick receptor for outer surface protein A), essential for spirochete colonization. OspA protein is then downregulated, while the tick prepares for the blood meal, the spirochetes passes from the midgut to the salivary glands and from there to the host. Simultaneously, Outer surface protein C (OspC) is upregulated (Schwan et al., 1995; Schwan & Piesman, 2002). The role of this protein is not clear, since it may have multifactorial activity including helping in host infection, invasion and dissemination, (Tilly et al., 2013). OspC expression and infectivity increases during some days once the

spirochetes have invaded host tissues by biding to the tick salivary protein, Salp15 (Pal et al., 2004; Pal & Fikrig, 2010) (Figure 17). Though being antigenic, it is eventually downregulated to minimize host antibody response.

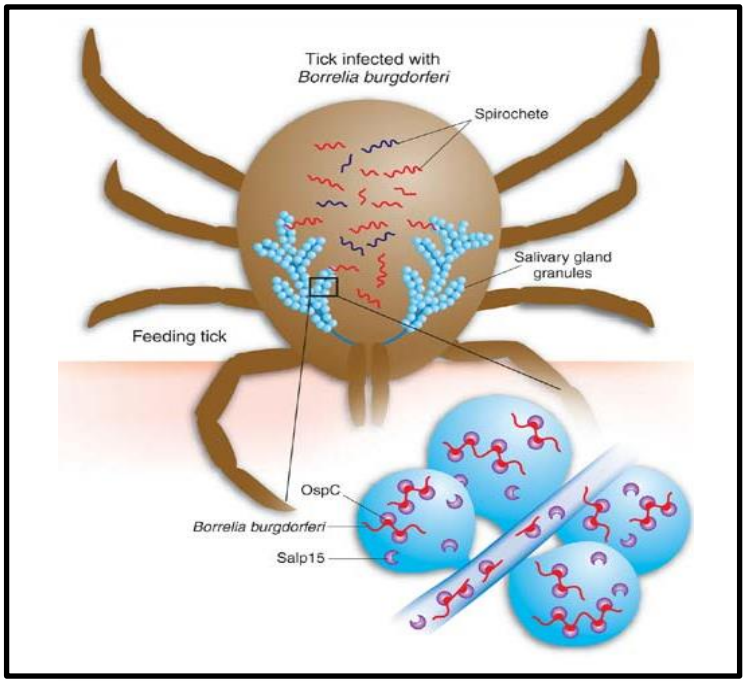


Figure 17 - Tick salivary protein (Salp15) that binds and protects *Borrelia burgdorferi* s.l. spirochetes. (Source: Rosa, 2005).

A major factor in the OspA/OspC complementary expression is the temperature. When a tick finds a host and starts to feed, it moves from ambient temperature to the temperature at the surface of mammalian host skin. This rapid temperature change influences the spirochete population and induces OspC expression (Schwan & Piesman, 2002). This can be important for *Borrelia* spirochetes transmission time from tick to host. Tick blood feeding behavior includes engagement, the adherence to the host; exploration, the search for a suitable site for attachment; and penetration, where the tick inserts the mouthparts in the host for feeding (Cook, 2015). During the process of tick exploration, temperature rise will activate OspA/OspC regulation and the process of increased motility and infectivity begins. Exploration time will be highly variable, since it depends on how

quickly the tick migrates to an optimal site. This time could vary with several factors as host animal size, competing ticks presence, or rejection of an unsuitable site (Cook, 2015).

In addition to ticks acquiring infections directly from an infected blood meal, as stated earlier, they can also get infected by a process known as cofeeding transmission. In this mode of transmission, uninfected ticks acquire infections from infected ticks that are feeding in close proximity to them on the same host. This phenomenon has been demonstrated in transmission of *B. burgdorferi* s.s. spirochetes by *I. scapularis* (Patrican, 1997; Piesman & Happ, 2001) and *I. ricinus* (Gern & Rais, 1996), *B. afzelii* by *I. ricinus* (Crippa et al., 2002), and *B. garinii* by *I. persulcatus* (Sato & Nakao, 1997). The significance of cofeeding transmission to the epidemiology of LD is poorly understood; however, it seems to be more efficient in the European "system" of *B. afzelii* and *I. ricinus* than the North American "system" of *B. burgdorferi* s.s. and *I. scapularis* (Voordouw, 2015). The importance is related to the potential for nymph-to-larva cofeeding events, which depends on the synchrony of larval and nymphal host searching and questing activity. In Europe, the two stages of immature ticks are active during the same times of the year from spring to autumn, whereas in North America, the peak activity of these stages may occur during different times of year (Kurtenbach et al., 2006; Barbour et al., 2009). Because deer and other large cervids may carry all stages of ticks simultaneously, these hosts may play an important role in providing a platform for cofeeding transmission to occur even though they are not infected themselves (Voordouw, 2015).

The host response to *B. burgdorferi* s.l spirochetes can also play a key role in disease pathogenesis. These bacteria does not produce toxins or proteases that are directly responsible for tissue damage upon colonization. In contrast, the bacterium produces multiple molecules that activate host responses and can lead to localized and generalized inflammatory pathogenic responses. Most of these host responses normally function to contain or clear infections and are components of the innate defense and/or inflammatory response (Benhnia et al. 2005; Behera et al., 2006; Oosting et al., 2010). Although their purpose is to clear infection, if continually activated, they lead to lesion development and disease.

One of these multiple molecules, are lipoproteins that activate Toll-like receptors (TLRs) 1 and 2 in a CD14-dependent manner (Hirschfeld et al., 1999), and also induces type I

interferon production in mice and human cells (Petzke et al., 2009). The adaptive immune response, particularly humoral response, is important in controlling the severity of LD in mice, but the bacteria can survive despite eliciting a strong antibody response, possibly because of immune evasion by modulating gene expression. In mice, a stronger initial T-helper type 1 (Th1) response seems to be important for controlling the infection (Zeidner et al., 2008). Interestingly, *Ixodes* tick saliva has been shown to induce a Th2 response that facilitates infection (Müller-Doblies et al., 2007).

1.4 - Reservoirs and hosts of *Borrelia* spirochetes

Borrelia burgdorferi s.l. transmission depends of a complex zoonotic cycle between hosts, reservoirs and vectors (Schwan et al., 2012). The typical and propitious habitat for spirochetes transmission is constituted by forest areas, with a humidity sufficient to support the development and survivor of the ticks, and by the presence of potential hosts/reservoirs (Stanek et al., 2012).

The capacity of an animal to serve as a reservoir host depends on four factors: *i)* presence of host vector ticks; *ii)* acquisition of the agent from infectious ticks; *iii)* capacity of the agent to multiply and/or develop and persist in its body and *iv)* transmission of the agent back to subsequently feeding vectors ticks (Stanek et al., 2002).

Ixodes ricinus ticks, has the capacity of feeding in more than 300 species of vertebrates, like rodents, birds and larger mammals (Figure 18) (Schwan et al., 2012). However, it seems that each *Borrelia* species is associated to a determined vertebrate, although this specialization is not absolute (Santos-Silva et al., 2011), this fact can have important implications in the spreading and dissemination of the different *Borrelia* species (Savic et al., 2012). Humans and domestic animals are considered accidental hosts, being infected with *B. burgdorferi* s.l. spirochetes when biting by infected ticks. Animals like dogs and horses can develop Lyme disease such as the humans, being dogs considered the sentinels of the disease (Richter et al., 2013; Vollmer et al., 2013).

Figure 18 – Examples of vertebrates where *Ixodes ricinus* ticks can take their blood meal. (Source: google images).

According to Radolf and collaborators (2012), a reservoir for *B. burgdorferi* s.l. consists in a vertebrate species that can be persistent and asymptomatically infected by the spirochetes, serving as a source of infection for the ticks that feeds on him (Radolf et al., 2012). Small mammals group have been the most investigated up to now in Europe, where *Apodemus sylvaticus*, *A. flavicollis*, *A. agrarius* and the vole, *Clethrionomys glareolus*, act as reservoirs for *B. burgdorferi* s.l., has been proved in several European countries (Humair et al., 1993; Gern et al., 1994; Kurtenbach et al., 1998; Hanincová et al., 2003; Piesman & Gern, 2004).

Depending on the *Borrelia* species, the reservoirs can be mammals, birds or reptiles, being evident that a variety of extrinsic ecological factors such as the climate, vegetation or geology, determine the relative role that a vertebrate host population plays in the maintenance and geographical distribution of a particular *Borrelia* species (Kurtenbach et al., 1995; Randolph & Craine, 1995). For example, *B. afzelii* is perpetuated by rodents, whereas *B. garinii* and *B. valaisiana* are associated mainly to birds, and *B. burgdorferi* s.s. is considered ubiquitous, being related to rodents and also to birds (Richter et al., 2013). Rodents also act as reservoirs of *B. bissettii* and *B. bavariensis*, while *B. lusitaniae* is maintained mostly by lizards (Schwarz et al., 2012; Richter et al., 2013; Vollmer et al., 2013). The role of reptiles, like the lizards, in *B. burgdorferi* s.l. maintenance seems to

vary with the species and the geographic area (Richter et al., 2013). Also the action of the complement system in lizards is extremely lytic for *Borrelia* spirochetes, and the ticks that feed in these vertebrates are less likely to transport the spirochetes, this is called the zooprophylatic effect (Richter et al., 2013). This type of speciation can be explain by the complement system present in the blood of the hosts, that showed to be an active component in the *Borrelia* host specificity (Kurtenbach et al., 1998). However, this explanation is still not clear, existing some exceptions like different associations that are found in nature, even in low frequency (Gern, 2008). Actually, several studies are contradictory to this fact, for example *B. afzelii*, *B. burgdorferi* s.s. and additional *Borrelia* species and strains were isolated, or its DNA detected, in small mammals (Faria et al., 2014; Leydet & Liang, 2014). Differences in biological and ecological factors such as different *Borrelia* strains, different tick vector species and different areas, could explain the discrepancy observed (Gern, 2008). Lyme disease analysis performed by a multilocus sequence analysis scheme (MLST), showed that OspA serotype 4 strain of *B. garinii* (rodent associated ecotype) were genetically distinct from the bird-associated *B. garinii* strains, resulting in a new species, *B. bavariensis* (Margos et al., 2009).

In the majority of *I. ricinus* tick habitats, the cervids, like deer (Figure 19), are essential for the maintenance of the vectors population, providing a blood source for infected and non-infected ticks to co-feed. Although the ticks show a special tropism to these animals, the cervid is not a competent reservoir for *Borrelia* spirochetes (Stanek et al., 2012). As such, the deer play a significant role in the amplification of tick populations, but are not themselves involved in the infectious life cycle of LD.

Figure 19 - Cervids represent a source for *Ixodes ricinus* feeding. (Source: Original photo by Mónica Nunes).

The presence of cervid populations in ticks habitats can be a good indicator of risk for Lyme infection, since other hosts, including competent reservoirs for *B. burgdorferi* s.l. can also be present (Stanek et al., 2012).

Finally, migratory birds revealed an important role in the distribution of *B. burgdorferi* s.l. spirochetes, since, besides being reservoirs of medical importance *Borrelia* species, can also act as vehicle of infected ticks, carrying and distributing them in long distances (Franke et al., 2013).

1.4.1 - Companion animals and Lyme disease

Lyme disease has been reported in dogs and also in horses (Wagner et al., 2012). Like to humans, dogs and horses are accidental, dead-end hosts for *B. burgdorferi* s.l. spirochetes. In contrast, small wild mammals (such as mice and squirrels) and possibly some migratory birds can act as reservoirs for maintaining the life cycle of *B. burgdorferi* between the tick vector and the host (Radolf et al., 2012).

Infection of companion animals living in close proximity to humans might be used as a sentinel for early detection of emerging LD helping to access the risk of *B. burgdorferi* s.l. spirochetes transmission to humans and animals in restricted geographical areas. Normally dogs are used as sentinels for emerging LD, by testing for antibody detection and seroprevalence to *B. burgdorferi* s.l. determination (Olson et al., 2000; Duncan et al., 2004; Hamer et al., 2009). For example, in one study, seroprevalence in dogs was determined by three conventional serological assays and was ineffective in tracking the spread of infected *I. scapularis* (Hamer et al., 2009). Moreover, in another study, a significant increase in seroprevalence was observed in dogs from endemic Lyme areas – supporting the potential of dogs as sentinels (Duncan et al., 2004). However, none of these serological studies could distinguish between recent and chronic infection, which reduces the timeliness of the sentinel for determination either of the emerging risk, or the continued incidence of infection with *B. burgdorferi* s.l..

Much less is known about LD in these animals comparatively to human. The cases of LD in dogs and horses are possibly underestimated, since the clinical signs can be non-specific. Also, subclinical infection can occur (Levy & Magnarelli, 1992). However,

many animals are now diagnosed as *B. burgdorferi* positive in endemic LD areas (like New York State), since there exists a higher awareness of owners and veterinarians to the disease, and also because better diagnostic methods are available for animal species. The most common clinical sign of LD in dogs is a migratory arthritis without divergent radiographic findings, but there is no erythema migrans characteristic of human LD. Less common clinical signs reported in dogs were carditis, glomerulonephritis, neuritis and renal lesions. Several studies aimed at showing a relation between renal disease and *B. burgdorferi* infection. ‘‘Lyme nephritis’’ was investigated in dogs with distinctive renal lesions and typical clinical signs, in which antibodies against *B. burgdorferi* were found (Gerber et al., 2009). Nevertheless, a causative role of *B. burgdorferi* in the development of renal disease was not confirmed, and renal lesions were not found in experimentally infected dogs (Summers et al., 2005). Because of the difficulties in finding sufficient indicative clinical signs, additional information about environmental life of dogs is of great importance in the clinical diagnosis of canine Lyme borreliosis (Krupka & Straubinger, 2010).

Nonetheless, LD diagnosis should be made based on clinical signs supported by an existing risk of infection (i.e. the animal lives or has been in a location with infected ticks) and a positive diagnostic assay for antibodies to *B. burgdorferi* s.l.. Most of these assays only indicates seroprevalence (not seroincidence). However, seropositive results do not necessarily indicate that an animal has or will develop LD, and neither seroprevalence nor seroincidence risks may be compared directly to human disease rates. Incident companion animal infections with *B. burgdorferi* s.l. nevertheless indicate that infected ticks were recently present.

In contrast to previous serological methods that could not distinguish between new infections and older, persistent infection with *B. burgdorferi* s.l., multiplex technology with its high analytical sensitivity, high-throughput capacity, and quick turn-around time (results can be reported as soon as the same or next day after receiving the sample) allows for a prompt evaluation of recent new infection by geographic region. This ability to identify new infections quickly in animals offers an opportunity of using animal infections for passive surveillance for infection risk of humans and additional animals (Briers et al., 2012).

1.5 – Lyme disease clinical manifestations, diagnosis and treatment

1.5.1 – Human clinical manifestations

Lyme disease is a complex multisystemic, infectious and chronic illness that can affect several organs and systems such as the skin, joints, musculoskeletal and nervous system, heart and eyes (Shamasna et al., 2012). Due to the diversity of clinical symptoms, LD is often considered in differential diagnosis (Wilske, 2005).This disease can be defined in three stages presented in Table 6 (early localized LD, early disseminated LD and late LD), however, it does not necessarily develop in these stages (Stanek et al., 2012).

Early localized LD (stage 1) – Characterized by the appearance of a slowly expanding skin lesion - erythema migrans, that consists in an annular skin rash (Table 6) that begins days to weeks after the tick bite, being the result of a local spreading of the spirochete through the skin (Reed, 2002; Heyman et al., 2010). This "bull's eye" affects about 70-80% of the patients being predominant in the upper and lower members and in the face, normally followed by series of flu-like symptoms such as headaches, fatigue, myalgia, malaise, arthralgia, fever and chills (Steere et al., 2004; Aguero-Rosenfeld et al., 2005; Strle & Stanek, 2009). This EM can be absent in cases of premature LD (Marques, 2010; Stanek et al., 2011).

Multiple EM lesions can also appear, commonly associated with several bites or hematogenous dissemination of spirochetes (Arnez et al., 2003).

Early disseminated LD (stage 2) – If in the stage 1 the treatment is not done, the infection spreads through the bloodstream and lymphatic system in weeks or months after the tick bite, causing systemic complications and specific symptoms such as:

- multiple EM that occurs in areas besides the tick bite site, or borrelial lymphocytoma (Table 6) that consists in a bluish red tumor-like skin infiltrate, up to a few centimeters

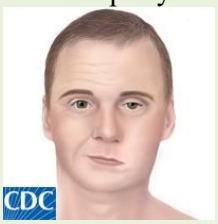
of diameter, with lymphoreticular proliferation in the dermis and/or subcutis (Mullegger, 2004);

- infection of the central nervous system (CNS) with the common manifestations of Lyme neuroborreliosis (LNB) including lymphocytic meningoradiculoneuritis – Bannwarth's syndrome, acute facial nerve palsy (Bell's palsy), which consists in a paralysis or weakness of muscles on one or both sides of the face (Table 6), and lymphocytic meningitis (Stanek & Strle, 2008; Mygland et al., 2010). LNB is an infectious disorder and the most frequent syndrome of disseminated infection on Europe, however, is becoming an more common symptom in North American LB patients (Garcia-Monco & Benach, 1998; Mygland et al., 2010);

- severe muscle pain or numbness in the arms and legs, being common pain or swelling in the knees, shoulders, elbows and other large joints. All these symptoms contribute to Lyme arthritis (Table 6) (Hu, 2005);

- a wide range of clinical cardiac complications, including palpitations and dizziness, atrioventricular block, pericarditis, myocarditis to more rare cardiomyopathy also known as Lyme carditis (Lelovas et al., 2008);

Table 6 - The three stages of Lyme disease and examples of some clinical manifestations.

Stage of disease	Timing	Common manifestations	Examples of clinical manifestations
Early Localized	Days to weeks	A solid red or bull's eye lesion (erythema migrans - EM); regional Lymphadenopathy.	EM 
Early disseminated	Weeks	Most commonly multiple rashes, Bell's palsy and meningitis; rarely carditis with varying degrees of heart block; also joint pain, headaches, stiff neck, lymphadenopathy, vision alterations.	Bell's palsy 
Late	Weeks to months	Recurrent arthritis; Acrodermatitis Chronica Atrophicans - ACA; neurological disorders; peripheral neuropathy.	ACA 

Chonic Late LD – Persistent infection (stage 3) – normally occurs months to years after the tick bite, with chronic manifestations of arthritis, acrodermatitis chronica atrophicans (ACA) (Table 6) and late neuroborreliosis manifestations including several degree of encephalopathy and encephalomyelitis, besides various neuropsychiatric symptoms.

ACA is associated to *B. afzelii* and usually begins on the extensor sites of the members extremities, on the lower leg with initial involvement of one foot, and it does not heal spontaneously (Stanek et al., 2002), being more common in Europe and Asia but not frequent in North American patients.

This diversity of symptoms, can be explain in part, by the different species of *Borrelia* responsible for LD in several geographic areas, and also possibly by genetic differences among the affected populations (Reed, 2002). For example in Europe LNB is most often caused by *B. garinii*, and skin complications are usually associated to *B. afzelii*, however, regarding the articular complications these can be due to several species like *B. garinii*, *B. afzelii* and *B. burgdorferi* s.s.. Meanwhile, in USA *B. burgdorferi* s.s. is the species responsible for cases of Lyme arthritis (Strle & Stanek, 2009), and the novel identified *B. burgdorferi* s.l. genospecies – *B. mayonii* – also causes Lyme borreliosis, but with substantially elevated spirochaetaemia and clinical features distinct from other recognized *B. burgdorferi* s.l. species (Pritt et al., 2016). Therefore, the clinical manifestations are distinct in North America and in Europe, reflecting the global distribution of the different spirochetes species and ever genotypes as further will be explain (Wang et al., 1999).

Furthermore, in Europe LD occurs in similar frequencies in both genders, with exception to ACA that is more common in women. Early LNB cases showed a bimodal age distribution with a lower frequency in the age range of 20 to 29 years old, while ACA occurs mostly in older patients (Wilske, 2005).

1.5.2 – Laboratory diagnosis – Conventional methodologies

Lyme disease diagnosis is mainly clinical, based in signs and symptoms, the patient's history of tick bite or exposure, anamnesis, and complemented by epidemiological data. In most cases the clinical diagnosis should be followed by laboratory tests, due to the unspecific nature of the clinical manifestations. The possibility that LD agents can be involve in other disorders, makes necessary for a laboratory response unequivocal, through sensitives and specific tests (Stanek et al., 2011).

Unfortunately, there are no standardized diagnostic criteria for LD, which has led to both over and under diagnosis of the disease. CDC has published case definitions for surveillance purposes, despite emphasizes that these are not intended as diagnostic criteria (Table 7) (CDC, 2011).

Table 7 - Case definition from Centers for Disease Control and Prevention (CDC), for surveillance purpose. (Font: adapted from Borchers et al., 2014).

Case definition	CDC
Erythema migrans	A skin lesion that typically begins as a red maculate or papule and expands over a period of days to weeks to form a large round lesion, often with central clearing. The largest diameter must reach a size ≥ 5 cm; The diagnosis must be made by a physician: Laboratory confirmation is recommend for person without known exposure.
Neuroborreliosis	Any of the following manifestations (alone or in combination): Lymphocytic meningitis; Cranial neuritis, particularly facial palsy (may be bilateral); Radiculoneuropathy; Encephalomyelitis; Encephalomyelitis must be confirmed by *B. burgdorfer*i -specific antibody production in CSF.
Musculoskeletal system	Recurrent brief attacks of objective joint swelling in one or a few joints, sometimes followed by chronic arthritis in one or a few joints.
Cardiovascular system	Acute onset of high-grade (2nd or 3rd degree) atrioventricular conduction defects that resolve in days to weeks and are sometimes associated with myocarditis.
Suspected	A case of EM without known exposure (defined as having been ≤ 30 days before the onset of EM in wooded, brushy, or grassy areas in a county in which Lyme disease is endemic); A case with laboratory evidence of infection but without available clinical information;
Probable	Any other case of physician-diagnosed Lyme disease that has laboratory evidence of infection;

(Cont. Table 7)	
Confirmed	A case of EM with a known exposure; A case of EM with laboratory evidence of infection and without a known exposure; A case with at least one late manifestation that has laboratory evidence of infection;
Laboratory evidence	Positive culture for *Borrelia burgdorferi* or Two-tier testing (for specific antibodies) interpreted using established criteria, where Positive IgM is sufficient during the first 30 days from symptoms onset; Positive IgG is sufficient at any point during illness; Single-tier IgG immunoblot seropositivity using established criteria; CSF antibody positive for *B. burgdorferi* s.l. by enzyme immunoassay (EIA), or indirect immunofluorescence assay (IFA), when the titer is higher than it was in serum.

Also the EUCALB (European Concerted Action on Lyme Borreliosis), has proposed clinical case definitions for use in clinical settings and epidemiological investigations (Stanek et al., 2011; Borchers et al., 2014). Except for EM, LD manifestations are not specific, having a variety of causes. Therefore, it is important to obtain a detailed patients history in order to establish probable exposure to *Ixodes* ticks in an endemic area at an appropriate time of the year, and to obtain appropriated and definitive laboratory confirmation.

Laboratory test have improved a lot in the last decades and clinicians have now available a range of options of methods that can be classified in two types: Direct methods (culture and molecular approaches), and Indirect methods (immunologic and serological approaches).

- *Direct methods*

Laboratory tests for direct detection of *Borrelia* are generally limited by the low number of spirochetes in clinical samples, also the lack of sensitivity of direct tests is one of the main challenges in the diagnosis of LD. Although direct tests for *B. burgdorferi* can be very helpful, none are usually required for the diagnosis of the disease (Marque, 2015).

The main direct test modalities used are culture and PCR for *Borrelia* DNA detection. Histopathology has limited utility, being used mostly to exclude other diseases, and in the evaluation of suspected cases of borrelial lymphocytoma and ACA (Müllegger & Glatz, 2008; Zajkowska et al., 2011). Detection of *B. burgdorferi* is difficult and time consuming because of the extreme scarcity of organisms (Duray, 1989; de Koning et al., 1995).

Culture

Culture is not a typically available diagnostic method for the diagnosis of LD in clinical practice, due to its relatively low sensitivity, long incubation time, requires special media and expertise. However, the ability to isolate and to maintain *B. burgdorferi* s.l. cultures is essential in research, and culture remains the gold standard to confirm the diagnosis. Methods that would improve sensitivity and simplify the procedure are needed to allow it to be adopted more extensively. Since *B. burgdorferi* s.l. has a limited metabolic capacity, a complex growth medium for cultivation is essential. The Barbour-Stoenner-Kelly medium (BSK) (Pollack et al., 1993) and the modified Kelly-Pettenkofer medium (MKP) (Ružić-Sabljić et al., 2006) are the most used medias for *B. burgdorferi* s.l. cultures. The BSK medium has the particularity of changing color, from orange to yellow, when the spirochetes growth, due to its acidification (Figure 20). Cultures can be examined using dark-field microscopy or fluorescent microscopy (Liveris et al., 2011). The spirochetes of *B. burgdorferi* s.l. species has a slow reproduction rate and cultures are maintained under anaerobic or microaerophilic conditions with a temperature ranging from 30-34ºC, during at least 8 to 12 weeks before being considered negative (Liveris et al., 2011).

Figure 20 - Cultures of *B. burgdorferi* s.l. in selective medium BSK. The changing of the medium color, from orange to yellow, shows the spirochetes growth. (Source: Original photo by Mónica Nunes).

The success of culturing *B. burgdorferi* s.l. depends of the specimen, the biological sample (e.g. fluids or biopsies), the evolution of the disease, and the expertise of the laboratory staff. It may also depend of the genotype (Xu et al., 2013). Also, if the patient was subjected to an antibiotic therapy with effective drugs against B. *burgdorferi* s.l. (even a single dose) the culture sucess rate is significantly affected (Nadelman et al., 1993; Picken et al., 1997).

Culture of skin biopsies from EM has a sensitivity of 40% to 60% (Liveris et al., 2012; Ogrinc et al., 2013; Ružić-Sabljić et al., 2014). In the USA, where disease is caused by *B. burgdorferi* s.s., positive cultures are associated with shorter duration of the disease and smaller lesions (Liveris et al., 2002; Li et al., 2011). In central Europe, positive skin biopsy cultures (mostly isolates were *B. afzelii*) were associated with larger lesions (up to about 15 cm in diameter) and increased duration (up to 30 days) (Strle et al., 2013). These findings are probably related with the different *Borrelia* species and the host immune response that eventually controls the infection. Culture is moderately successful in skin biopsies of ACA lesions (Picken et al., 1997).

Culture of plasma samples from untreated patients with early disseminated infection has a sensitivity of around 40%, which can be increased to 75% by frequent testing culture aliquots with a sensitive PCR. Blood cultures are more likely to be positive in patients with multiple EM (Liveris et al., 2011). *B. burgdorferi* s.l. is rarely cultured from the blood of LD patients with later manifestations (Nowakowski et al., 2009; Maraspin et al., 2011). Isolation of *B. burgdorferi* s.l. from other origins, as CSF and synovial fluid is uncommon and the isolation rate is very low reflecting the small number of viable organisms present in those locations (Wormser et al., 2012).

Microscopy

The spirochetes can be directly detected in biologic samples such as blood, tick tissues and skin biopsies by dark-field microscopy, staining with appropriate stains, and by histochemical techniques. However, due to the low number of spirochetes in samples and to the limitations of microscopy observation, this approach is rarely used (Baptista, 2006).

Polymerase chain reaction (PCR)

In the last decades, many laboratories have started to give more attention to the molecular assays, with the aim to increase the sensitivity and specificity of LD diagnosis, and reduce the time consuming of the conventional techniques. The detection of *Borrelia* DNA carried through PCR presents a variable sensitivity, ranging from 10-30% (in case of CSF samples), to 50-70% for blood, skin biopsy and synovial fluid samples (Bratton et al., 2008; Stanek et al., 2011). These variation is due to the methodology, gene targets and primer sets used (Picken et al., 1997; Glins et al., 2008).

Conventional PCR or nested-PCR can be used, yet nested-PCR in more specific and sensitive since it uses two steps of amplification, with one set of primers each, instead of the single step and single pair of primers involve in the classical PCR. Several targets have been used for the amplification of *B. burgdorferi* s.l. DNA, such as 5S/23S rDNA intergenic region, 16S genes, *flagellin* and *p66* chromossomal genes or the *ospA* and *ospB* genes (Priem et al., 1997; Schmidt, 1997; Wilske et al., 2007). The targets carried on plasmids (*opsA, ospB, opsC* and *vlsE*) are present in multiple copies within each bacterium, and assays with these targets presents a greater sensitivity than those using single-copy chromosomal targets such as *flagellin*, *recA*, *rpoB*, 16S and 23S rDNA, and intergenic spacers.

Restriction Fragment Length Polymorphism-PCR (RFLP-PCR), is a derivation of the PCR, and it is normally used for genotyping *B. burgdorferi* s.l. species, being the most used target the intergenic region 23S (*rrl*) – 5S (*rrf*) of the rDNA, were the amplification product is then digested by an endonuclease (*Mse*I or *Dra*I), and an pattern of products with different sizes is obtained, allowing to differentiate between *Borrelia* genospecies (Postic et al., 1994).

Other PCR-based techniques can be used for the direct diagnosis of LD, such as Multiplex Real-Time PCR and Reverse Transcriptase PCR (RT-PCR) (Limbach et al., 1999; Courtney et al., 2004), however, a standardized PCR protocol is yet to be defined (Wilske et al., 2007).

A negative result in a PCR test cannot be interpreted as an exclusion of LD. Since the number of spirochetes in infected tissues or in body fluids of patients are very low, and appropriated procedures for sample collection, transport and DNA extraction are critical

for reliable and consistent PCR results (Wang et al., 2010). The false positive results is one of the limitation of nucleic acids amplification methods, due to contaminations, which can be very troublesome in assays set for maximum sensitivity, a requirement for LD diagnosis (Schmidt, 1997).

- *Indirect methods*

The host immune response to *B. burgdorferi* s.l. can be detected by indirect methods, based on the presence/absence of antibodies in serum against the spirochetes. Only the antibody-based assays are approved and recommended for Lyme disease testing by the USA Food and Drug Administration (FDA) (Marques, 2015).

In USA about 3.4 million of Lyme serologic tests are done per year, almost 1000x more than the estimated number of 300,000 cases of LD, and a major problem of these laboratory tests is its inappropriate use. Most likely these tests are being used in conditions for which they are not recommended, including ruling out LD in populations with a low probability of having the disease. The predictive value of a test is determined by its sensitivity, specificity, and the prevalence of LD in the tested population. Therefore in a patient with a low probability of LD, a negative tests rules out the disease, whereas a positive result is more likely to be a false-positive (Marques, 2015).

Already in 1995 the CDC recommended for LD test performance and interpretation a standardized 2-tier testing (STTT) approach to improve the specificity of serologic tests in USA (Figure 21) (CDC, 1995).

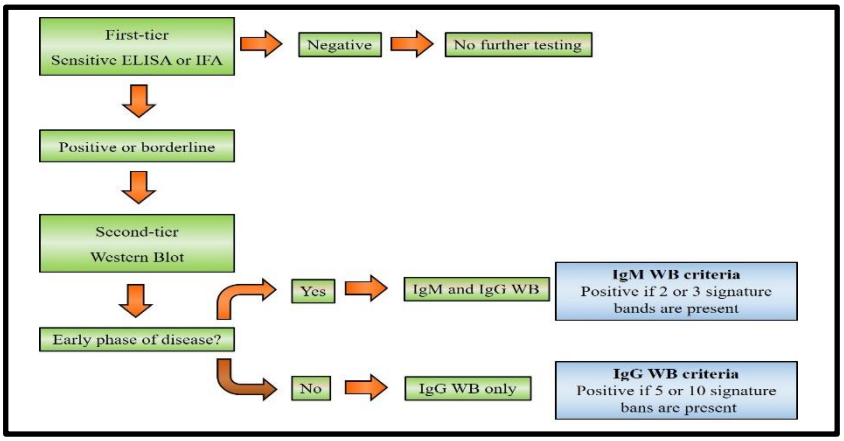


Figure 21 - Current CDC recommendations of serologic diagnosis of Lyme disease. (Source: Adapted from Marques, 2015).

The serum samples should be tested with a sensitive first-tier EIA, or IFA, and if the result is borderline or positive, an IgM and IgG Western blot (WB; also called immunoblot) is applied as second step (Marques, 2015; Schriefer, 2015). Later on it was stipulated that IgM WB should only be applied to patients in an early phase of the disease, with a duration of 30 days or less. The WB is interpreted by a standardized criteria that requires at least two or three antigenic fractions (signature bands) for a positive IgM WB (p21 [OspC], p39 [BmpA], and p41 [flagellin B] (Engstrom et al., 1995), and five to ten antigens for a positive IgG WB (p18, p21 [OspC], p28, p30, p39 [BmpA], p41 [flagellin B], p45, p58, p66, p93 (Dressler et al., 1993). The 2-tier approach algorithm has a good performance when used as recommended, however, there's still many improvements to be done, including the situations of low sensitivity during early infection, subjective interpretation of bands, and difficulty of health care providers in interpreting the results (Marques, 2015).

The majority of indirect assays is based on whole-cell sonicate (WCS) from *B. burgdorferi* s.l. cultures, however, a significant number of false-positive results can occur, due to cross-reactive antigens (Gomes-Solecki et al., 2000). Moreover, some antigen expression can differ from culture to *in vivo*, for example the expressed VlsE lipoprotein that causes a strong humoral response during infection, has a minimal expression in cultured *B. burgdorferi* s.l.. The addition of this lipoprotein to the 2-tier approach has improved its performance (Branda et al., 2010). Also, tests that use C6 peptide (26-amino acid peptide from conserved region 6 of VlsE has a sensitivity similar to WCS-based EIAs, with significantly improved specificity (Branda et al., 2011; Wormser et al., 2013). Several others recombinant and synthetic antigens have been evaluated in serodiagnosis of LD, including antigens combining portions of different proteins (Arnaboldi et al., 2013).

Antibody-based test sensitivity increases with the evolution of the infection, and there is a lag from initial infection until the time when there are sufficient levels of antibodies to be detected. Patients who present very early symptoms are more likely to have a negative result for LD. Less than 50% of patients with EM are seropositive at presentation, and these patients should receive treatment based mainly on the clinical diagnosis.

Serologic tests are most helpful in patients with clinical findings indicating later stages of LD. As shown in many studies, the additional IgM WB step decreases sensitivity in early disease, the unique situation in which its use is recommended (Steere et al., 2008; Branda et al., 2010; Wormser et al., 2013). Positive IgM results for *Borrelia* can occur in more than 40% of parvovirus B19 infections (Tuuminen et al., 2011) and have also been observed in human granulocytic ehrlichiosis patients (Wormser et al., 1997), Epstein-Barr virus infections, and patients with autoimmune diseases. Therefore, the algorithm testing for early LD should be changed to avoid the use of the IgM WB. Possible strategies include the use of the WCS ELISA followed by the C6 ELISA (Branda et al., 2011; Wormser et al., 2013), the addition of the VlsE band (Branda et al., 2010), and the use of multipeptide assays (Arnaboldi et al., 2013).

Finally the current assays do not distinguish between active and not active infection, and patients may continue to be seropositive for years, showing an IgM response, even after adequate antibiotic treatment (Kalish et al., 2001; Peltomaa et al., 2003). Studies in recent years have proposed new immunoassays including the combination of multiple antigens, to help in the early diagnosis, to inform on the stage of LD, and on the presence of active versus past infection (Chandra, et al., 2011).

1.5.3 – Molecular-based strategies for the assessment of the *B. b*urgdorferi s.l. species

Over the past century microbiologists search for rapid and efficient means of microbial identification. For many years the identification and differentiation of microorganisms have principally relied on microbial morphology and growth variables. Advances in molecular biology over the past 10 years have opened new opportunities for microbial identification and characterization (Tang et al., 1997). Methods such as nucleic acid amplification tests (NAATs) and mass spectrometry, are two of the most common approaches that are being adopted into clinical laboratories for routine detection and identification of microorganisms. Also, hybridization tests, that can be performed on microarrays or biochips, which can have thousands of single-stranded nucleic acid samples arranged in a grid on a solid surface. This allows screening of a clinical sample

for many different nucleic acid sequences at the same time in an automated system (Cobo, 2012).

The nucleic amplification tests generally fall into one of three categories: target amplification systems, such as PCR; probe amplification, such as DNA ligase chain reaction (LCR); and signal amplification for example, branched DNA (bDNA) technology. Most of these assays can provide results in a couple of hours, allowing a rapid identification of microbial sequences that confer drug resistance and can potentially overcome some of the shortcomings of traditional culture-based methods (Goldenberg, 2013).

- *DNA amplification-based assays*

Molecular tools for *Borrelia* detection can be developed in regions where epidemiological, clinical or serological data are available. However, this approach to laboratory diagnosis of LD, may be complicated by the recognition of multiple distinct species of *Borrelia* that have been associated with LD in humans (Schriefer, 2015). With international travel becoming increasingly common, the clinical laboratories may be asked to identify LD from patients whose infections are caused by *Borrelia* species not endemic in the local area. NAATs can, however, serve as an supplementar diagnostic modality alongside with clinical findings and serologic testing and, depending on the specimen source, has a good performance in cases of acute disease (Swanson et al., 2006; Ivacic et al., 2007; Maraspin et al., 2011).

In an early infection, NAATs may serve to confirm the diagnosis when the antibody response is undetectable by serologic methods. Also, in later stages of disease or in cases of suspected reinfection, particularly when serologic methods may not be able to establish the diagnosis, NAATs offer high diagnostic specificity (Alby & Capraro, 2015).

Most NAATs known for the detection of *B. burgdorferi* s.l. DNA have used a variety nucleic acid-based techniques, including end-point PCR, nested PCR, real-time PCR, Loop-mediated isothermal amplification (LAMP), and sequence-based identification, among others. In these techniques, the target gene ideally is sufficiently conserved to allow the amplification of multiple species of *Borrelia* but sufficiently different to allow

discrimination between species (Nolte, 2012). Primers define the specificity of the PCR and the sensitivity is influenced by reaction parameters such as annealing temperatures, concentration of primers, quality and quantity of template DNA and the presence of inhibitors.

There are several PCR protocols to enhance detection sensitivities, especially when dealing with small numbers of bacterial cells as targets. Nested PCR is one of these approaches, allowing the detection of only a few bacteria in clinical specimens. As mentioned before in section 1.5.3, the process utilizes two consecutive PCRs. The first PCR contains an external pair of primers, while the second contains either two nested primers that are internal to the first primer pair, or one of the first primer and a single nested primer –semi-nested PCR. The larger fragment produced by the first reaction is used as the template for the second PCR (Mothershed & Whitney, 2006). The sensitivity and specificity of DNA amplification can be considerably improved by using such nested PCR strategies, sometimes being one thousand times more sensitivity than a standard PCR.

Most conventional first-generation PCR assays involved complex procedures for detecting the amplification products (e.g. gel electrophoresis and ethidium bromide staining). A great technological advance towards the simplification of nucleic acid testing assays was the development of quantitative real-time PCR - qPCR, whose main advantage, over the traditional PCR assays, is that the starting DNA concentration is determined with accuracy and high sensitivity. Thus, the obtained results can be either qualitative (showing the presence or absence of the DNA sequence of interest) or quantitative (allows the extrapolating back to the starting DNA concentration). In contrast, conventional PCR is, at best, semi-quantitative. Moreover, the amplification reactions are run and data are analyzed in a closed-tube system, eliminating the need for post-amplification manipulation and therefore reducing opportunities for contamination (Longo et al., 1990; Kaltenboeck & Wang, 2005).

The ability to monitor the progress of DNA amplification in qPCR depends on the chemistry of the reaction and instrumentation used. Normally, chemistries consist of special fluorescent probes that must associate a fluorescent signal to the amplification of DNA (Monis & Giglio, 2006). The initial qPCR procedures used the double-stranded (ds)

DNA-specific intercalating dye ethidium bromide (Higuchi et al., 1992). DsDNA-specific dyes exhibit little or no fluorescence when free in solution, but produce a strong fluorescence signal when bound to dsDNA and exposed to the appropriate wavelength of light. There is a wide variety of commercially available fluorescent DNA dyes, including ethidium bromide (Higuchi et al., 1992), YO-PRO-1 (Ishiguro et al., 1995), SYBR® Green I (Ririe et al., 1997), SYBR® Gold (Tuma et al., 1999), SYTO (Monis et al., 2005; Gudnason et al., 2007), BEBO and BOXTO (Bengtsson et al., 2003), and EvaGreen (Wang et al., 2006). These dyes can be used to detect either single or two or more different DNA sequences in a single PCR reaction (multiplex assays).

However, the use of these dsDNA binding dyes has the disadvantage of detecting not only specific products, but also nonspecific products and primer-dimers produced during the qPCR reaction, which may cause problems with the accuracy of qPCR. Additionally, SYBR Green is the most commonly used binding dye (Figure 17), despite its popularity, it presents some limitations, including limited dye stability and dye-dependent PCR inhibition (Monis et al., 2005; Mao et al., 2007).

Because of these limitations, other strategies that involve the use of probes that will specifically recognize only if the target PCR product have been developed. The most common probes currently used in real-time PCR assays are the *TaqMan* probes, the molecular beacons and fluorescence resonance energy transfer (FRET) probes (Espy et al., 2006).

TaqMan probes are one of the most widely used approach because the assay design is relatively simple and generally robust. This system and Minor Grove Binder (MGB) probe technologies both work by a similar principle, where they have a fluorescent reporter molecule, and a quencher molecule. *TaqMan* probes are typically 20–30 nucleotides in length and the fluorescence of the reporter molecule is quenched at this proximity between reporter and quencher molecule (Figure 22). Hydrolysis of the *TaqMan* probe by the exonuclease activity of Taq DNA polymerase at 60ºC separates the reporter and quencher molecule and a signal is emitted by the reporter molecule that is detected by the real-time PCR instrument (Figure 22).

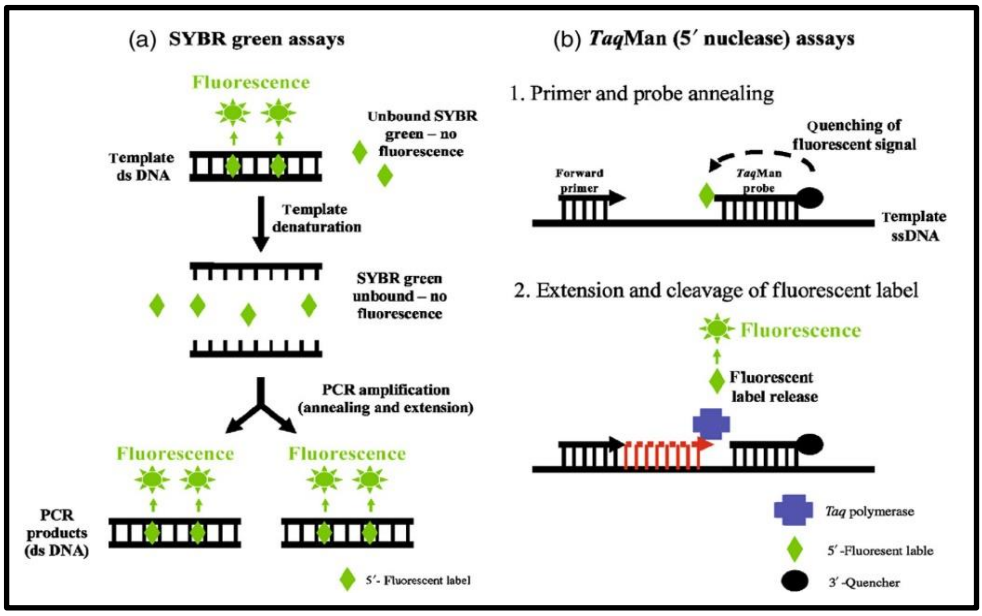


Figure 22 - Schematic representation of SYBR Green and *TaqMan* chemistries: (a) SYBR green detection; (b) *TaqMan* assay using *TaqMan*® probes. (Source: adapted from Smith & Osborn, 2009).

The incubation temperature of DNA synthesis step is critical because at higher temperatures (e.g. 72 ºC), which are normally used for DNA synthesis in conventional PCR, the Taq DNA polymerase will displace the probe rather than degrade it. As a result of probe degradation, fluorescent signal increases as a function of the number of amplification cycles and allows specific detection and quantitation of the target DNA (Monis & Giglio, 2006). These probes can also be used for performing DNA melting curve analysis (by measuring the dissociation kinetics of the release of the bound probe to the target DNA), allowing a further genetic characterization of the amplified DNA.

TaqMan probes do not allow confirmation that the correct fragment has been amplified (other than by running a gel), and as a result, it is particularly important to validate the specificity of primer/probe combinations. Multiplexing *TaqMan* assays is achieved using probes labelled with different reporter molecules that have distinct fluorescence properties (namely excitation/emission maxima). The combinations of fluorophores that can be used is dependent on the technical specifications of the real-time PCR instrument used (an upper limit of six fluorophores can be detected on some instruments). *TaqMan*

probes allow presence/absence detection of a particular target sequence but do not allow genetic discrimination unless used in a multiplex format.

Molecular beacons (Saidac et al., 2009) and MGB Eclipse probes (Afonina et al., 2002) both use secondary structure (a stem-loop in the case of molecular beacons) to hold a reporter-molecule and quencher in close proximity when the probe is in solution for MGB, preventing the production of any fluorescent signal. When either type of probe anneals to target-DNA, they unfold and there is sufficient distance between the reporter and quencher molecules to allow fluorescence.

Regarding the fluorescence resonance energy transfer (FRET)-based assays, these relay on the energy transfer between a 3′-end donor fluorophore and a 5′-end reporter fluorophore on separate probes, rather than the quenching of a fluorophore as seen with molecular beacons and *TaqMan* probes (Didenko, 2001; Monis & Giglio, 2006). Melt curve analysis in FRET assays measures the temperature at which the bound probes are dissociated from the target amplicon and not the melting temperature of the entire amplicon as is the case when using intercalating dyes. Thus, a single primer set may be used to amplify a region of interest, with species/strain discrimination possible by designing the FRET probes to bind to variable regions within the region bound by the primer set used for amplification. This technology lends itself to single nucleotide polymorphisms (SNPs) detection, as a single mismatch between the probe and target sequences will yield a sufficiently different melting temperature to allow detection (Navarro et al., 2014).

In the last decades, numerous methods have been described for the molecular detection and identification of *Borrelia burgdorferi* s.l., using for example PCR approaches (Malloy et al., 1990; Guy & Stanek, 1991; Rijpkerna et al., 1997; Sato et al., 1997; Schmidt, 1997; Ornstein et al., 2002; Ranka et al., 2004), and several real-time amplification chemistries (Rauter et al., 2002; Fu et al., 2012; de Leeuw et al., 2014). The molecular approach to laboratory diagnosis of LD is complicated due to the recognition of multiple distinct species of *Borrelia* that have been associated with LD in humans, as previously mentioned.

Besides the technology selected, the DNA targets for amplification are also varied. *Borrelia* species contain both chromosomal and plasmid DNA, and molecular assays have

been investigated using a variety of targets from both types of DNA (Schmidt, 1997; Aguero-Rosenfeld et al., 2005).

Assays designed to target plasmid-borne genes, such as *ospA*, *ospC*, or *vlsE*, are more sensitive than those targeting chromosomal, *flagellin* or 16s rDNA genes (Persing et al., 1994; Zore et al., 2002), most likely because of the finding that *Borrelia* often shed plasmid-containing blebs, which allows for higher concentrations of plasmid than chromosomal DNA. However, it is now well recognized that these blebs disassociate from the spirochete and may persist in tissues and body fluids (Persing et al., 1994). Therefore the detection of plasmid DNA from these nonviable blebs may elicit false-positive results that do not necessarily reflect ongoing LD. Chromosomal targets usually occur as single copies; although targeting these genes may result in lower analytical sensitivity, they may be a better predictor of organism viability (Liveris et al., 1999).

Several studies show that some matrices are better than others for the direct detection of *Borrelia* DNA from clinical specimens. The performance of these specimens in NAATs depends on the stage of infection at the time of patient presentation. Although these assays have demonstrated high specificity, sensitivity has been lacking, probably due to the absence of a true gold standard assay, or a standardized approach for comparison of the various methods under development. However, NAATs can serve as an adjunct diagnostic modality alongside with clinical findings and serologic testing (Swanson et al., 2006; Maraspin et al., 2011).

- *Isothermal DNA amplification*

In the last decade, it has been observed a huge rise in the abundance and availability of nucleic acid information, allowing the use of DNA and RNA amplification techniques for specific detection, harnessing the complexity inherent in genetic material for the purpose of targeted identification. Molecular diagnostic techniques using nucleic acids were pioneered through use of PCR, which remains the predominant method in the field due to its robustness, sensitivity and familiarity. However, the growing use of these molecular diagnostic methods has emphasized speed and simplicity as key criteria for adoption in point-of-care and field applications, and isothermal amplification techniques are well-suited for these uses. Due to their nature, isothermal amplification methods require only

a single temperature, avoiding the need of costly thermal cycling equipment and potentially even electrical power, depending on incubation temperature and heating (Tanner & Evans, 2014). Moreover, by constant incubation and amplification, no temporal restrictions from defined cycles are implied, resulting in amplification reactions as rapid as fifteen minutes (Fang et al., 2010; Wang et al., 2011).

The most widespread isothermal method is loop-mediated isothermal amplification (LAMP), where since its first publication in 2000 by Notomi and collaborators, these technique has been applied to diagnostic detection of hundreds of pathogens in clinical, plant, food and animal samples (Arai et al., 2015; Ferrara et al., 2015; Palacio-Bielsa et al., 2015). This methodology presents a simple, robust and flexible platform for molecular diagnostics.

The LAMP reaction employs a DNA polymerase with strand displacement activity and four or six specially designed primers that recognize six distinct sequences on the target DNA under isothermal conditions (60-65°C), where a denatured template is not required. Normally, the reaction runs for about 60 minutes, showing an extremely high specificity (Nagamine et al., 2002; Mori & Notomi, 2009). Also, LAMP method has a high amplification efficiency that allows the synthesis of large amounts of DNA in a short time. Its detection limit is a few copies per reaction and therefore is comparable to PCR (Mori & Notomi, 2009). For the assay performance, only a heating block at a constant temperature or a water bath is necessary.

To perform the reaction, a set of two specially designed inner and outer primer pairs and a DNA polymerase with strand displacement activity are required for the DNA synthesis. The initial reaction steps are illustrated in Figure 23.

DNA regions F3 and R3 are complementary to F3c and R3c on the template, respectively. The F2 region in the forward inner primer FIP is complementary to the F2c region followed by the F1c complementary to F1 of the target DNA. The same principle is used to design the backward primer. As a result, these four primers recognize six distinct sequences which ensure high specificity for target amplification. Moreover, these primers enable generation of a stem-loop DNA for subsequent complex LAMP cycling including self-priming reactions. In the initial steps of the LAMP reaction all four primers are employed, but in the later cycling steps, only the inner primers are used for strand

displacement DNA synthesis. The final products is a mixture of stem loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats in the same strand (Notomi et al., 2000; Tomita et al., 2008). The reaction can be accelerated by using two extra loop primers.

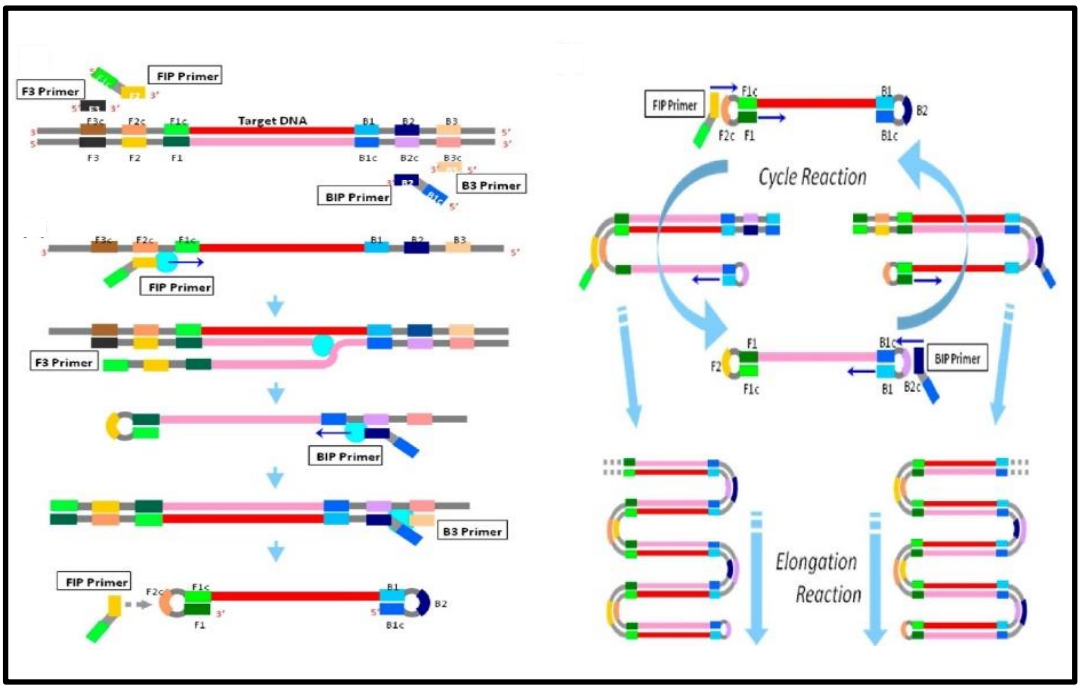


Figure 23 - Schematic representation of Loop-mediated isothermal amplification assay. LAMP is characterized by the use of four primers (F3, B3, FIP and BIP), in an isothermal (60–65ºC) auto-cycling strand displacement reaction. (Source:http://what-when-how.com/tropical-medicine/novel-molecular-diagnostic-platform-for-tropical-infectious-diseases-other-tropical-infectious-and-non-infectious-conditions-part-1).

LAMP amplification products can be detected either by gel electrophoresis, real-time monitoring of turbidity with a turbidimeter (Mori et al., 2001; Mori et al., 2004), or simply with the naked eye. During the reaction, a large amount of DNA is synthesized, yielding a large pyrophosphate ion by-product. It was observed that pyrophosphate forms an insoluble, observable white precipitate with divalent metallic ions (Mori et al., 2001). Another visual detection method based on the formation of pyrophosphate can be accomplished by using the fluorescent metal indicator calcein, which binds free calcium ions. Calcein has been used for the real-time detection of DNA formation during LAMP (Tomita et al., 2008). Further methods apply intercalating DNA dyes such as SYBR

Green I (Soliman & El-Matbouli, 2005), FDR (Yoda et al., 2007), or oligonucleotide probes labeled with different fluorescent markers, as well as low molecular weight cationic polymers such as polyethylenimine (Mori et al., 2006).

There are several works reporting the use of this technology to detect pathogenic organisms, however, for *Borrelia* this approach is still hardly applied, existing so far only two published studies (Yang et al., 2013; Zhang et al., 2015).

It's important to employ LAMP technique on large scale in resourced-limited laboratories in developing countries, where many fatal tropical diseases are endemic. Also in the near future, LAMP testing kits on readymade microchips are to be used by both developed and developing countries.

- *Immunochromatographic assays*

In the late 1960s immunochromatographic assays were first described, being originally developed to assess the presence of serum proteins (Kohn, 1968; Peruski et al., 2003). However, over the past decade many other applications have been developed for immunochromatographic assays, including the detection of bacterial pathogens (van Dommelen et al., 2008; Preechakasedkit et al., 2012; Widiyanti et al., 2013).

The instantaneous examination of changes in one's own physical symptoms or health status is increasingly preferred. Self-tests performed at home will definitely be an integral part of future health care systems (Price, 2001). In fact, the market expansion of home-version diagnostic kits in developed countries, typically in the United States, far exceeds the average for overall *in vitro* diagnostic products. The first commercially successful kit was the pregnancy test based on the rapid detection of human chorionic gonadotropin in urine by simply adding urine to the test kit (Butler et al., 2001).

The most common immunochromatographic assays are the known lateral flow strips that have been a commonly used technology for some time. Lateral flow strips offer a number of various benefits including user friendly format, very short test time, long term stability, and they are producible at low costs. These features make the lateral flow strip tests ideal for home testing, rapid point of care testing and for field testing applications.

A lateral flow strip (LFS) presents four main sections made of different materials, as shown in Figure 24: sample pad, made of cellulose, where the sample is dropped; conjugate pad, made of glass fiber, impregnated with the bioconjugates solution (the label particle and a receptor for the analyte); detection pad, a nitrocellulose (Ahmad et al., 2009) where test line (TL) and control line (CL) are printed; and absorption pad, also made of cellulose.

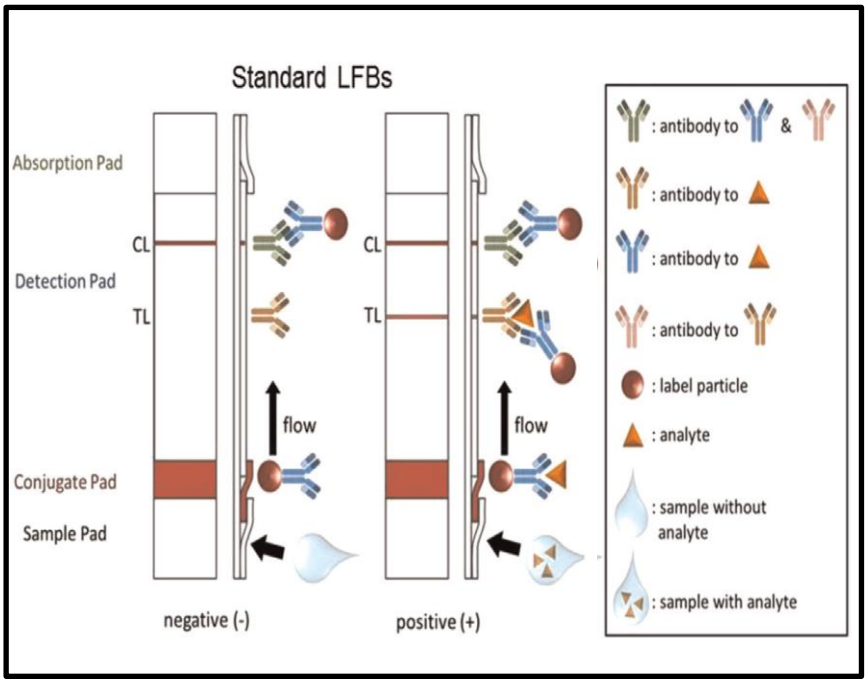


Figure 24 - Schematic representation of a LFS (lateral flow strip) and movement of analytes and label particles across it. (Source: Adapted from Quesada-González & Merkoçi, 2015).

Other additional parts can be integrated on LFS as blood filters, substituting the sample pad, to retain big particles like red blood cells and avoiding their hemolysis. Another example of material which can be integrated on LFS is carbon nanotubes paper, with high conductive properties to connect LFS to electronic devices (Zhu et al., 2014).

The principle of an assay with a LFS is simple: the sample is added on the sample pad and then the liquid will start flowing to the conjugate pad where the analyte, if present on the sample, will be linked to the transducers (the label particles), previously conjugated

with a bioreceptor specific to the analyte. The conjugate, rehydrated by the liquid, will flow by capillarity forces across the detection pad to the absorbent pad, passing through the TL, where it will be captured only if the conjugate has the analyte attached (positive response), and to the CL, being always captured, evidencing that the assay worked (Figure 24) (Quesada-González & Merkoçi, 2015).

LFSs can be used to detect a large range of biomarkers that may include not only proteins, but also nucleic acids and even whole cells, among other biocompounds. Furthermore, LFSs are not limited only to biomolecules detection; several publications have appeared in the last years about the detection of pollutants such as metallic ions, pesticides, etc. The range of LFSs applications is including detection of hazardous (Shyu et al., 2002), heavy metals in drinking waters (López-Marzo et al., 2013), allergens and pathogens in food (Berlina et al., 2013), pesticides (Wang et al., 2009), drugs screening (Inoue et al., 2007), etc.

These tests are, therefore, of great value in situations where health professionals need to make decisions and take immediate measures. Lateral-flow assays were also previously described for the diagnosis of LD (Lerner et al., 2013).

1.5.4 – Prevention, Control and Treatment

Given the increasing threat of LD, the need for effective methods to protect against this disease has never been greater (Ogden et al., 2013). The options for this purpose are limited, since there are no licensed human vaccines against Lyme disease and also an area-wide and centrally organized tick control programs are lacking (Poland, 2011). However, exposure to ticks and *B. burgdorferi* s.l. can be controlled, usually at the individual person or individual property level, with several relatively simple interventions (Poland, 2001; Corapi et al., 2007; Piesman & Eisen, 2008):

- Avoiding areas where ticks that transmit *B. burgdorferi* s.l. occur, at times that the ticks are active;
- Applying personal protective measures, such as wearing appropriate clothing, using tick repellents and clothing treatments, and removing ticks before they can attach and transmit *B. burgdorferi* s.l.;

- Reducing environmental risk by controlling ticks and tick infections with pesticide applications; reservoir-targeted interventions (e.g., bait boxes); and landscape management;
- Using prophylactic antibiotics in an appropriate manner after a tick bite to prevent transmitted *B. burgdorferi* s.l. from evolution to clinical LD.

A simple rule for LD is: "if you don't get a tick, you don't get sick." Previously this rule could be achieved just by avoiding the areas where LD occurs, however, the range expansion of *Ixodes* species and LD in USA and Europe has changed this, and these ticks are now found in more regions and have also moved into more densely populated areas, including on or close to private/residential properties (Stanek & Reiter, 2011; Li et al., 2012; Medlock et al., 2013; Vollmer et al., 2013). Nonetheless, avoidance can be a viable risk-reduction approach, at least in some locations and situations.

If it's not possible to avoid the tick habitats, then risk reduction relies on preventing bites or remove attached ticks before they have time to transmit *Borrelia*. This can be accomplished by wearing appropriate clothing, like light-colored and long-sleeve shirts, socks, and full trousers; or use approved, topical repellents like DEET (N,N-diethl-meta-toluamide) and permethrin based products, on skin or wear insecticide-treated clothing; do tick checks at least once a day and remove any ticks that are found with fine-tipped, stiff, and angled forceps (tweezers) placed around the head of the tick as near as possible to the skin, followed by a steady, upward pulling movement (Figure 25) (Piesman & Dolan, 2002; Duscher et al., 2012); finally bath or shower soon after leaving tick habitat, within the two following hours.

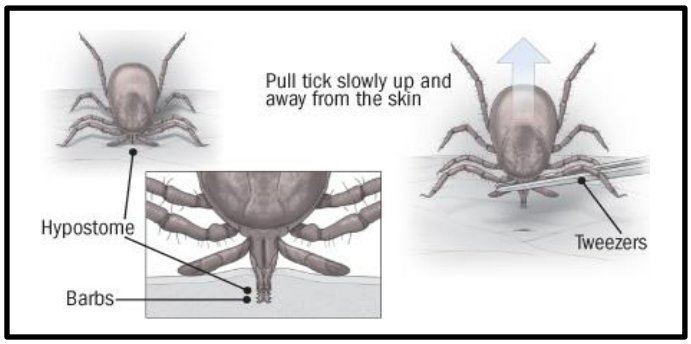


Figure 25 - Scheme showing how to remove a tick.
(Source: adapted from http://www.health.harvard.edu/blog/matchless-strategy-for-tick-removal-6-steps-to-avoid-tick-bites 201306076360).

LD vaccination is still a problem, since there is no licensed human vaccine. Although, some studies defend that this disease can be prevented by vaccination with the OspA (Edelman et al., 1999; Rahn, 2001). A vaccine targeting LB (LYMErix) based on OspA from *B. burgdorferi* s.l. was tested, and show to be effective, being available in the USA from 1998 to 2000 (Golde et al., 1995; Steere et al., 1998). However, the duration of this vaccine was relatively short, and it was removed from the market by its manufacturer in 2002. Currently, efforts are being made towards the development of a broadly protective LB vaccine. Several proteins have been assessed as potential vaccine candidates. (Marconi & Earnhart, 2010; Comstedt et al., 2015).

Regarding the treatment of LD, this is routinely with antibiotics; therapy hastens the resolution and largely prevents the development of other disease manifestations. The classes of antibiotics that have shown the greatest effectiveness against *Borrelia* spirochetes are b-lactams (in particular cephalosporins) tetracyclines and, to a lesser extent, macrolides. The best treatment approach, in particular the duration of therapy, is a matter of ongoing debate. It is quite evident that not all patients, and most certainly not all species or strains of *Borrelia* respond equally to the antibiotics most commonly used in the treatment of LD (Preac-Mursic et al., 1996). Based on the available evidence from randomized controlled trials, treatment recommendations have been published by the Infectious Diseases Society of America (IDSA) (Wormser et al., 2006), the American Academy of Pediatrics, and by a variety of national and supranational associations in Europe (Mygland et al., 2010; EUCALB). Both guidelines published by the IDSA and the EUCALB, are similar on both sides of the Atlantic regarding the approaches to therapy, yet there are some differences in the recommended dosage and treatment duration.

1.6 – Objectives and thesis plan

In Portugal LD still remains underdiagnosed and underreported. Despite the existence of the vector in the country and the identification and isolation of several genospecies of *B. burgdorferi* s.l. from patient samples and from the vector, the true prevalence of the

disease is still unknown. However, an increase importance has been given to this disease worldwide, which is currently considered an emerging disease.

The diagnosis it is mainly clinic, although the laboratory information based in serologic or molecular assays, is a fundamental support contributing for an adequate and timely treatment, and at the same time, helping to limit the risk of resistances to treatments or the evolution of the infection to a chronic situation, resulting in high costs for the patient and for the community.

Objectives

The main goals of the present study were to evaluate the prevalence of LD agents in the Portuguese ixodofauna, mainly in *I. ricinus* vector and in sylvatic and domestic hosts; and to develop two new molecular methodologies for the identification of four of the most prevalent genospecies of *B. burgdorferi* s.l. in Europe. Thus, this thesis was divided into four main parts:

1) To evaluate the bio-ecological characteristics of the ixodids collected in the select districts from Portugal;

2) To analyze vector-pathogen-host relationships as they happen in nature, therefore gaining insight into the diversity and prevalence of *B. burgdorferi* s.l. organisms in different hosts and tick species;

3) To develop and optimize a real-time PCR assay for the identification and quantification of four of the most prevalent genospecies of *B. burgdorferi* s.l. in Europe/Portugal;

4) To develop two duplex Loop-Mediated Isothermal DNA Amplification Assays (dLAMP) coupled with colorimetric lateral flow devices, for the identification of four of the most prevalent genospecies of *B. burgdorferi* s.l. in Europe/Portugal;

Thesis plan

This dissertation is organized into six chapters. In **chapter 1**, a general theoretical introduction embracing the subject under study is presented, with emphasis to the topics regarding the major characteristics of the *B. burgdorferi* s.l. complex members, and its laboratory diagnosis; **chapter 2** characterize bio-ecologically the ticks as vectors,

collected from the vegetation and hosts in previously selected districts from mainland Portugal, and determined the infection rate by *B. burgdorferi* s.l.; in **chapter 3** the vector-pathogen relationships in nature are analyzed; in **chapter 4** the pathogen-host relationship is evaluated; in **chapter 5** the development of a rapid identification real-time PCR algorithm for *B. burgdorferi* s.l. complex species using specific dual-labelled hydrolysis probes in a multiplex format are described, and also two duplex Loop-Mediated Isothermal DNA Amplification assay (dLAMP) coupled with colorimetric lateral flow devices, for the identification of four of the most prevalent genospecies of *B. burgdorferi* s.l.. Finally, in **chapter 6**, considerations about the work presented and the main results that were achieved are highlighted, as long as suggestions for future work.

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Chapter 2

Distribution and bio-ecological characterization of ixodids in selected areas of Portugal: *Borrelia burgdorferi* s.l. infection

2. Distribution and bio-ecological characterization of ixodids in selected areas of Portugal: *Borrelia burgdorferi* s.l. infection

Ticks are obligate parasites, considered to be second worldwide to mosquitoes as vectors of human diseases, but they are the most important vectors of disease-causing pathogens in domestic and wild animals. The important role that ticks play in maintaining and transmitting tick-borne pathogens in Portugal, reinforces the need to offer an up to date summary of the information on ticks, their biology, ecology and associations with vertebrate hosts. Also, a better knowledge of *B. burgdorferi* s.l. infection rate in these arthropods, mainly in the vector *Ixodes ricinus*, is important in order to determinate possible risk areas for human and veterinary health. Therefore, this chapter the distribution and characterization of ixodids will be addressed in nine districts of Portugal, previously selected, alongside with their infection rate by *B. burgdorferi* s.l. using two nested-PCR.

This chapter is based on the research paper:

Nunes M, Vieira ML, Lopes N, Maia C, Almeida APG. 2016. Characterization and distribution of hard-ticks in nine districts of mainland Portugal where *I. ricinus* presence was previously reported: *Borrelia burgdorferi* s.l. prevalence.

(in submission)

2.1 Characterization and distribution of hard-ticks in nine districts of mainland Portugal where *I. ricinus* presence was previously reported: *Borrelia burgdorferi* s.l. prevalence

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Abstract

Several changes in spatial distribution and abundance of tick species and their associated pathogens have occurred in the last years, due to climate change, habitat modifications and globalization of human activities. Consequently, it's increasingly important to update tick distribution, biology, ecology and association with hosts and pathogens. In Portugal, there are favorable climatic conditions to the maintenance of ticks and their pathogenic agents. An example of that are the spirochetes of *B. burgdorferi* s.l. complex, Lyme disease (LB) agents, whose main vector in Europe is the tick *Ixodes ricinus*. Although this disease is underdiagnosed and underreported, several studies have confirmed the circulation of these spirochetes in tick populations from different areas of Portugal. Thus, the aim of this study was to collect and identify ticks from hosts and vegetation, in nine districts of Portugal, previously identified as areas with both the vector and the pathogen, and to determine *B. burgdorferi* s.l. infection rate, contributing to update tick's fauna and LB epidemiology.

Questing ticks were collected in seven of the surveyed districts, being *Rhipicephalus sanguineus* the most widespread species, although, in Lisboa *Ixodes ricinus immature* were the most abundant species. Regarding the hosts, pets (dogs and cats), sylvatic (cervids and wild boars), and livestock (cattle, sheep and donkeys) animals were surveyed, from seven districts, and again *R. sanguineus* was the most widespread species, except in Lisboa and Évora districts, where *I. ricinus* and *R. bursa* were the most abundant species, respectively. Regarding *B. burgdorferi* s.l. infection rate, 8% and 1% of the collected ticks were positive, at vegetation and host level, respectively. *Borrelia burgdorferi* s.l. positive ticks were collected in Braga, Vila Real, Lisboa, Setúbal, Évora and Faro, six of the nine surveyed districts, showing that this pathogen presents a general distribution throughout the country.

Changes in the distribution of ticks and their invasion into new regions were observed in this study, possibly related to changes in the landscape, climate and vegetation, to which ticks are very sensitive. Also the frequent large-scale movements of humans and their animals may be speeding up the introduction of novel tick species and their associated pathogens that can have severe consequences in human and animal health. Therefore, more studies concerning

tick distribution and behavior should be carried out in order to understand the biological mechanisms underlying successful tick invasions and adaptation to new local conditions leading to a successful establishment.

Introduction

Ticks (Ixodida) are obligate hematophagous arthropod ectoparasites with a worldwide distribution, and responsible for transmitting pathogens that cause diseases in humans and animals (de La Fuente & Contreras, 2015), leading to public health issues and economical losses in livestock production (Parola & Raoult, 2001). The incidence of tick-borne diseases (TBDs) is increasing worldwide, since ticks are capable of transmitting disease-causing protozoa, bacteria and viruses. For instance, more than 250 000 human cases of Lyme disease were reported in the last decade in the USA (http://www.cdc.gov/lyme/), and in Europe about 50 000 cases are reported each year in humans (Piesman and Eisen, 2008). This is due to the continuous human exploitation of environmental resources and to the increase of human outdoor activities that allow contact with ticks normally present in natural habitats (de La Fuente & Contreras, 2015). Furthermore, the expansion of tick populations due to climate changes and human interventions that affect reservoir hosts mobility and human contact with infected ticks, is a growing problem (Gray et al., 2009; Estrada-Peña et al., 2012; Otranto et al., 2015; Ostfeld & Brunner, 2015).

These arthropods extend from the tropics to subarctic areas and are well adapted to living in strict and diversified habitats, seeking hosts to feed upon, digesting blood meals, and developing through different life stages to adulthood, and further reproducing (Magnarelli, 2009). Although most ticks have close relationships with vertebrate animals, some species have limited host preferences, while others have broad host ranges.

Several studies have been carried out to understand tick species distribution, bio-ecological preferences and host-vector-pathogen relationships in different settings. Faunistic studies across several regions, namely the Mediterranean region, are of great importance and interest to characterize the distribution and composition of tick species affecting livestock, as a preliminary step to the knowledge of the pathogens they may transmit, and the economic

effects of these on animal production, and in public health (Papadopoulos et al., 1996; Bouattour, 1999; Estrada-Peña & Santos-Silva, 2005).

Portugal, the westernmost country in continental Europe, presents a total of 92 090 km^2 of land surface, 3.4 million hectares correspond to forested areas, mainly localized in the North of the Tagus river, with agroforestry and forest grazing areas localized in the South of the country (Figure 1).

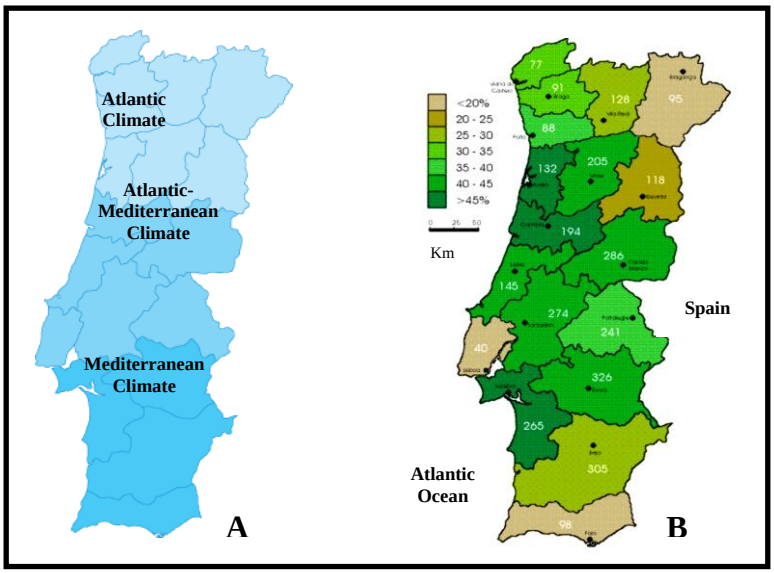

Figure 1 - Schematic representation of Atlantic Ocean and Mediterranean Sea influence in the Portuguese climate (A) and the Portuguese districts rate of forestation (B). (Source: de Macedo, 1997).

According to Koeppen-Geiger classification, Portugal has a temperate continental climate, Type C, checking the subtype Cs (a temperate climate with dry summer) and the following varieties: Csa, temperate climate with warm summer and dry in the interior regions of the Douro Valley (part of the district of Bragança), as well as in South regions of the mountain system Montejunto-Estrela (except on the west coast of Alentejo and Algarve); Csb, temperate climate with dry and mild summer, in almost all regions of the Northern mountain system Montejunto-Estrela and the regions of the west coast of Alentejo and Algarve. In a small region of Alentejo, in the district of Beja, is Arid Climate - Type B Subtype BS (steppe climate), BSK variety (cold steppe climate of mid-latitude).

The ecological, climatic and environmental conditions of Portugal are favorable to the development and maintenance of several hard-tick species that can transmit a large variety of pathogenic agents able of causing diseases in humans and animals, with an emergent risk in the country. For example, the tick *Ixodes ricinus*, competent vector of *B. burgdorferi* s.l. agents, is present in several regions within the country with different climate types and land cover (Núncio et al., 1993; Baptista et al., 2004; Estrada-Peña & Santos-Silva, 2005; Baptista, 2006).

In this study nine districts representative of North, Lisboa Tagus Valley (LTV), and South regions of mainland Portugal were selected for tick collections, based on data from previous studies, where *I. ricinus* presence was registered (Baptista, 2006; Santos-Silva et al., 2011). Also, the collected ticks were surveyed for *B. burgdorferi* s.l. DNA, to further characterize the infection rate with this pathogenic agent across the country.

Material and Methods

Characterization and location of sampling sites

A total of nine districts were selected: Braga, Vila Real, Aveiro, Guarda, (considered North region for comparison purposes), Santarém, Lisboa (considered Lisboa and Tagus Valley-LTV), and Évora, Setúbal and Faro (considered South region), (Figure 2). Ticks were collected from the vegetation and from several hosts present in the chosen areas.

Figure 2 – Map of mainland Portugal showing the districts where tick collections were performed (in green).

In the different districts, collections of questing ticks in the vegetation was carried out in several habitats (Figure 3), and more emphasis was given to the forest areas with biotic factors (hosts) and abiotic factors (vegetation and habitat types), humidity, temperature, elevation and distance to waterline) favorable to the development of *Ixodes ricinus*. The characterization of each district is presented in Table 1.

Figure 3 – Examples of habitats where ticks were collected: **A –** Amares (Braga)**; B** – Mondim de Basto (Vila Real); **C** – Dunas de São Jacinto (Aveiro); **D** – Guarda; **E** – Santarem; **F** – Tapada Nacional de Mafra (Lisboa); **G** –Grandola (Setúbal); **H** – Alcaçovas (Évora); **I** – Serra de Monchique (Faro).

Table 1 – Characterization of each surveyed district regarding the area, climate and counties.

District	Characterization of the district
Braga	Localized in the Northwest of the Iberian Peninsula, with 183.2 Km^2 of area. The climate is temperate Atlantic, due to its location between the mountains and the Atlantic Ocean. The relative humidity rounds the 80%, allowing the maintenance of the annual medium values of temperature between 12.5 ºC and 17.5 ºC (www.cm-braga.pt/). In this district tick collections were carried out in eight sites within Braga, Celorico de Basto, Cabeceiras de Basto, Vieira do Minho and Amares counties (Figure 3A).
Vila-Real	Localized in the North region with 378.80 km² of area. Its climate is temperate Mediterranean, but with a transition to the maritime climate, since the annual medium temperature is about 13.5ºC and the precipitation higher than 1000 mm. Due to its geographic situation where the Marão and Alvão mountains act like natural barriers, Vila Real presents a climate with some continentality (www.cm-vilareal.pt/). Tick collections were performed in four sites, namely Mondim de Basto, Ermelo, Varzea de Ermelo and Rebordelo (Figure 3B).
Aveiro	Localized in the Center region, with an area of 197.58 km². This city is limited by the Ria de Aveiro, and a relatively narrow range of coastline on the Atlantic Ocean in the west. The climate is Oceanic temperate with an influence of the Mediterranean, being a transition zone of Mediterranean temperate climate to the oceanic climate. Tick collections were carried out in the nature reserve of Dunas de São Jacinto, limited by the west of Atlantic Ocean and the east of Ria de Aveiro (www.icnf.pt/portal/ap/r-nat/rndsj) (Figure 3C).
Guarda	Localized in the center region of Portugal, with 712.1 km² of area in the Northeast of Serra da Estrela, being the tallest city of the country, with 1056 meters (m) of altitude. The climate is temperate with Mediterranean influence. It is considered one of the coldest city with temperatures in the Winter of about 4ºC or less, which sometimes leads to snowing (www.mun-guarda.pt). Tick collections were performed in two sites within Guarda county (Figure 3D).
Santarem	Integrates the Alentejo region and Lezíria of Tejo, with 552.54 km² of area. The climate is between the maritime, with influence of the Atlantic air, and the attenuated continental, although with more specific characteristics in the more elevated areas (www.cm-santarem.pt/). Tick collections were

	(conti. Table 1)
	carried out in three sites, namely Benavente, Albergaria and Almoster (Figure 3E).
Lisboa	Localized in the South with 100.05 km^2 of area, is the capital of Portugal and the most crowded city of the country. The climate is Mediterranean, strongly influence by the Gulf Stream. Tick collections were carried out in fourteen sites within Azambuja, Loures and Lisboa counties. However, the majority of the collections, were made at Tapada Nacional de Mafra (TNM), a protected ecological and recreational area with 819 hectares of dense forests very rich in fauna and flora (Figure 3F). The diversity of habitats present in TNM (woods, grasslands, brush and water courses), enable the existence of a large number of animal hosts, mainly fallow-deer's, wild boars, some carnivores and innumerous species of small rodents (http://tapadademafra.pt/en/), (Figure 3F).
Setúbal	Localized in the Metropolitan area of Lisboa with 230.33 km² of area. The climate is clearly Mediterranean with hot and dry summers, and mild but rainy Winters, where the humidity is high. Only the Arrábida area, due to its elevated altitude and the proximity to the sea, benefits of a more Atlantic climate (www.mun-Setúbal.pt/). Tick collections were performed in six sites within Almada, Grandola and Palmela counties (Figure 3G).
Évora	Localized in Alentejo region in the South of Portugal, with 7.393 km² of area. The climate is typical Mediterranean with Atlantic influence where the precipitation is distributed along the year in an uneven way. Évora presents drier environmental conditions, with arid lands and high temperatures in the summer. (www.cm-evora.pt/). Tick collections were carried out in four sites within Montemor-o-novo, Viana do Alentejo, Évora and Pegões counties (Figure 3H).
Faro	Capital of Faro district, former province of Algarve region, with 202.57 km² of area. The climate is Mediterranean with mild temperature all over the year (www.cm-faro.pt/). Tick collections were performed in seven sites within Faro, Castro Marim, Portimão, and more often, Monchique counties. In Monchique the captures were carried out in the mountain with 902 m of altitude, one of the most prominent points of Portugal (739 m). Due to the proximity to the sea, the climate is subtropical humid which associated to mild temperatures allows the existence of a rich and varied vegetation with rare species of trees (www.cm-monchique.pt) (Figure 3I).

Tick collection

Collection in the vegetation

From May 2012 to May 2014 collections of questing ticks were carried out in 23 sites across Portugal by two methods: *flagging* in the higher vegetation, by using one square meter flannel with a stick attached to the leading edge and used like a flag; and *dragging* in the lower vegetation and in the ground, by using a 1m x 1,5m towel also with a stick attached to the leading edge, but pulled by a string (Vassallo et al., 2000; Dantas-Torres et al., 2013; Dantas-Torres & Otranto, 2013; Richter et al., 2013) .

The collections were made from Spring to Autumn non-systematically (opportunistically), with the knowledge, from preliminary studies, that the peak season for tick abundance was from March to July. Thus, each sample site was surveyed at least once during the years 2012 to 2014. Although, in Lisboa tick collection took place more often from May 2012 to May 2014 at TNM, due to its proximity to Instituto de Higiene e Medicina Tropical (IHMT), Grupo de Leptospirose e Borreliose de Lyme (GLBL) laboratory. Also the collections were performed during different periods of the day and different periods of time, depending on the climate conditions. In each collection a field form (Supplementary Data - Fig.1) was filled regarding the number of the collection, the local, the type of vegetation, and the environmental variables (temperature, humidity, precipitation, wind, visibility). The temperature and humidity were registered with a thermo-hygrometer (HI 9564 *HANNA instruments*) at the beginning and at the end of each collection.

Collection on hosts

Tick collections on hosts were carried out during the same period in 26 sites, according to the presence and availability of animals in the sampling sites. The surveyed hosts were pets (dogs and cats), sylvatic (cervids and wild boar), and livestock (cattle, sheep and donkeys) animals. Each host was examined by observation and palpation of some strategic areas like the ears, eyes, neck, anogenital and abdominal regions. Ticks were removed with tweezers in order to keep the mouth parts intact. Some of the collections were made by other collectors, and therefore very few environmental variables were possible to register.

Tick maintenance

All ticks were properly stocked in dry plastic tubes with a screw cap, to which was added a piece of vegetation and small holes where made in the cap, to allow ventilation, keeping the ticks alive until arrival to the GLBL laboratory.

Ticks were examined with a binocular stereomicroscope (Motic SMZ-168) and identified to species level, using taxonomic keys and illustrations (Santos-Dias, 1994; Estrada-Peña, 2000; Estrada-Peña & Santos-Silva, 2005; Pérez-Eid, 2006). Furthermore, ticks were separated according to the collection site, species, stage and gender, and kept at 4ºC for further DNA extraction. Alongside, an identification form (Supplementary Data – Fig.2) was filled with all the referred variables.

DNA extraction

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

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

Detection of *B. burgdorferi* s.l. DNA was carried out using two different nested-PCR protocols. One of them targeted the intergenic spacer region (IGS), located between the 5S and 23S rRNA, using the 23SN1 and 23SC1 external primers (which amplify a 380 bp DNA fragment), and the 23SN2 and 5SC inner primers (which amplify a 225 bp DNA fragment), as described by Rijpkema et al., (1995). The nested-PCR protocol used included a denaturation step at 94.5ºC for 1 minute, 25 cycles of amplification at 94ºC for 30 seconds,

52ºC for 30 seconds (outer primers), or 55ºC for 30 seconds (inner primers), and 72ºC for 1 minute, followed by a 5 minute extension phase at 72ºC. The second nested-PCR protocol used, targeted the flagellin gene (*flaB*) (Wodecka et al, 2010). This included a first amplification reaction based on the use of outer primers 123f and 905r (which amplify a 774 bp DNA fragment), with a second amplification step using inner primers 220f and 824r (yielding an amplification product of 605 bp). The PCR conditions included an initial denaturation at 94ºC for 10 minutes, followed by 40 cycles of amplification, including denaturation at 94ºC for 30 seconds, annealing for 45 seconds at a temperature dependent on the primers used (outer primers-50ºC; inner primers-54ºC). An additional elongation step was carried out at 72ºC for 1 minute with a final elongation at 72ºC for 7 minutes. PCR protocols were done in a separate vertical laminar flow bench using a different set of micropipettes, for PCR use-only as well as filtered tips and sterilized material to ensure a contamination-free environment. *B. garinii* DNA was used as positive control and ultrapure water as negative control.

Nested-PCR products were detected by electrophoresis in 1.5% agarose gels stained with GreenSafe Premium (NZYTech), and visualized under UV light, using a Dolphin-1D Gel Image Analysis Software (Wealtec®) equipment.

Tick and bioecological data processing, and statistical analysis

Tick densities were estimated for each district and tick species. Both the total ticks collected, and the ticks of each species, were estimated regarding effort unit, *i.e.* collector-min for the questing ticks from the vegetation, or per animal surveyed, in the case of ticks collected from hosts. The arithmetic mean and the standard deviation were calculated for densities per region (North, LTV and South), season and year for all collections. Tick densities were analysed regarding bioecological and environmental variables mentioned above.

Statistical analysis was carried out using the SPSS package version 23.0 for Windows (SPSS for Windows ® Inc). Kolmogorov-Smirnov (Lilliefors modification) and Shapiro-Wilk tests were used to analyse data for normality, while Levene's test was used to test for homogeneity of variance. Due to the lack of normality of the data, large standard deviations and lack of

homogeneity of variance, non-parametric tests were used to analyse tick densities (Siegel & Castellan, 1988). Kruskal-Wallis (KW) tests were used for comparing, tick densities between years, regions, seasons, vegetation types, habitats and elevation categories. Whenever significant differences were found ($P < 0.05$), multiple comparisons were performed using the Dunn-Bonferroni (DB) pairwise comparisons. Spearman's's rhô non-parametric tests were used to study possible correlations between registered tick densities and continuous variables, such as temperature, humidity and distance to the waterline.

Differences in *Ixodes ricinus* tick infection rates among each district, were compared using Chi-squared test and Fisher's exact test.

Results

Ticks collected from the vegetation

A total of 4251 ticks were collected during the two-year period, corresponding to a total of 169.80 hours-collector, resulting in very low tick densities (0.00 – 0.80 ticks/min-collector). The three stages were collected, being 1058 larvae (25%), 1118 nymphs (26%), 1138 females (27%) and 937 males (22%) (Table 2). Also, 11 species from five different genera were identified, being *I. ricinus* the most frequent species (1681, 39.5 %), followed by *R. sanguineus* (1637, 38.5%), *H. punctata* (404, 9.5%), *Hy. lusitanicum* (192, 4.5%), *D. marginatus* (172, 4%), *R. bursa* (73, 1.7%), *H. inermis* (43, 1%), *Hy. marginatum* (37, 0.9%), *D. reticulatus* (5, 0.1%)l *R. boophilus* (4, 0.1%) and *I. hexagonus* (3, 0.1%).

Lisboa district showed the highest number of collected ticks (2634, 61.9%), followed by Faro (474, 11.0%), Setúbal (415, 9.8%), Aveiro (239, 5.6%), Braga (210, 4.9%), Évora (147, 3.5%) and Vila Real (132, 3.1%).

Densities of the several tick species collected in each year, district and season are represented in Figure 4, being *R. sanguineus* the most abundant species collected in all districts, except in Lisboa, which was *I. ricinus* species. For a more accurate analysis the distribution of tick species was analyzed by regions, being the graphics in different scales for a better comprehension of tick densities.

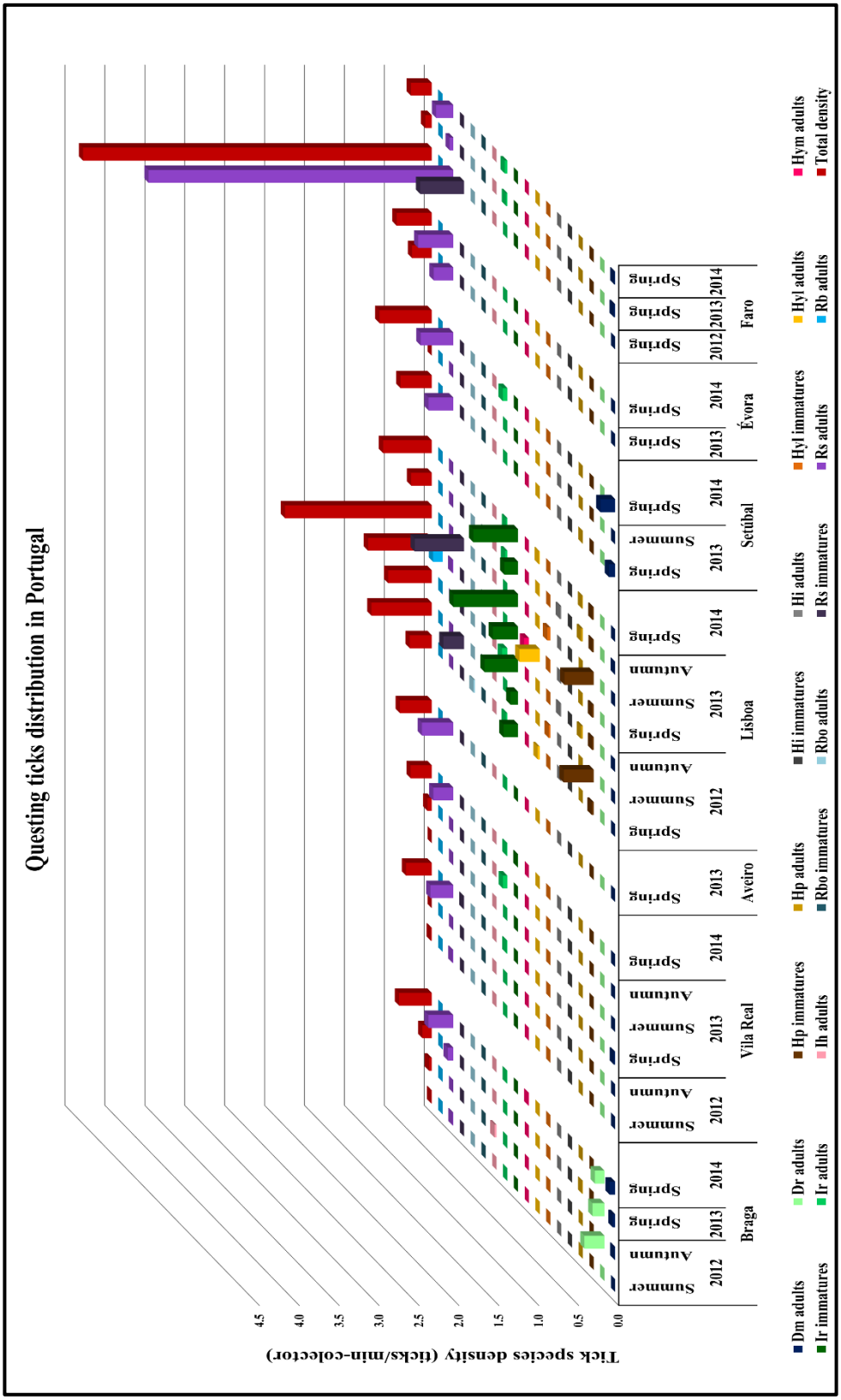

Figure 4 – Questing tick species average density for each collected species by season, year and district. (Dm – *Dermacentor marginatus*, Dr – *Dermacentor reticulatus*, Hp – *Haemaphysalis punctata*, Hi – *Haemaphysalis inermis*, Hyl – *Hyalomma lusitanicum*, Hym – *Hyalomma marginatum*, Ir – *Ixodes ricinus*, Ih – *Ixodes hexagonus*, Rbo – *Rhipicephalus boophilus*, Rs – *Rhipicephalus sanguineus*, Rb – *Rhipicephalus bursa*).

Table 2 – Total questing tick species by stage collected in each district.

		Ticks collected from the vegetation				
		Stage				
Districts	**Tick species**	**Larvae**	**Nymphs**	**Females**	**Males**	**TOTAL**
Braga	*Dermacentor marginatus*			26	15	41
	Dermacentor reticulatus			2	3	5
	Haemaphysalis punctata		1	2	3	6
	Hyalomma marginatum			1	0	1
	Ixodes ricinus			4	0	4
	Ixodes hexagonus			3	0	3
	Rhipicephalus sanguineus			77	73	150
	Total		**1**	**115**	**94**	**210**
Vila Real	*Dermacentor marginatus*			3	1	4
	Ixodes ricinus		1	7	8	16
	Rhipicephalus bursa				1	1
	Rhipicephalus sanguineus		2	65	44	111
	Total		**3**	**75**	**54**	**132**
Aveiro	*Ixodes ricinus*				3	3
	Rhipicephalus sanguineus			141	95	236
	Total			**141**	**98**	**239**
Lisboa	*Dermacentor marginatus*			7	1	8
	Haemaphysalis inermis	1		26	16	43
	Haemaphysalis punctata	259	76	22	39	396
	Hyalomma lusitanicum	8	21	107	56	192
	Hyalomma marginatum			1	35	36
	Ixodes ricinus	490	1011	43	60	1604
	Rhipicephalus boophilus	1		1	2	4
	Rhipicephalus bursa			38	33	71
	Rhipicephalus sanguineus	271	1	6	2	280
	Total	**1030**	**1109**	**251**	**244**	**2634**
Setúbal	*Dermacentor marginatus*			65	35	100
	Haemaphysalis punctata				1	1
	Ixodes ricinus			12	7	19
	Rhipicephalus sanguineus			165	130	295
	Total			**242**	**173**	**415**
Évora	*Rhipicephalus sanguineus*			83	64	147
	Total			**83**	**64**	**147**
Faro	*Dermacentor marginatus*			13	6	19
	Haemaphysalis punctata				1	1
	Ixodes ricinus			17	18	35
	Rhipicephalus bursa			1		1
	Rhipicephalus sanguineus	28	5	200	185	418
	Total	**28**	**5**	**231**	**210**	**474**
	TOTAL	**1058**	**1118**	**1138**	**937**	**4251**

Questing Ticks in the North region

In the North region (Figure 5), *R. sanguineus* was the most prevalent species, during the two years of collections, particularly in the spring. The species *D. reticulatus* was only collected in this region (Braga district) during summer of 2012 and spring of 2013 and 2014, being always collected in the same site, which was a hunting area with wild boars nearby. Also, *I. hexagonus* was only collected in Vila Real during the 2012 autumn in a site with high elevation, situated in the natural park of Alvão. Regarding Aveiro district the collections were only made once in the spring of 2013 near the seaside at Dunas de São Jacinto, where *R. sanguineus* was the most abundant species.

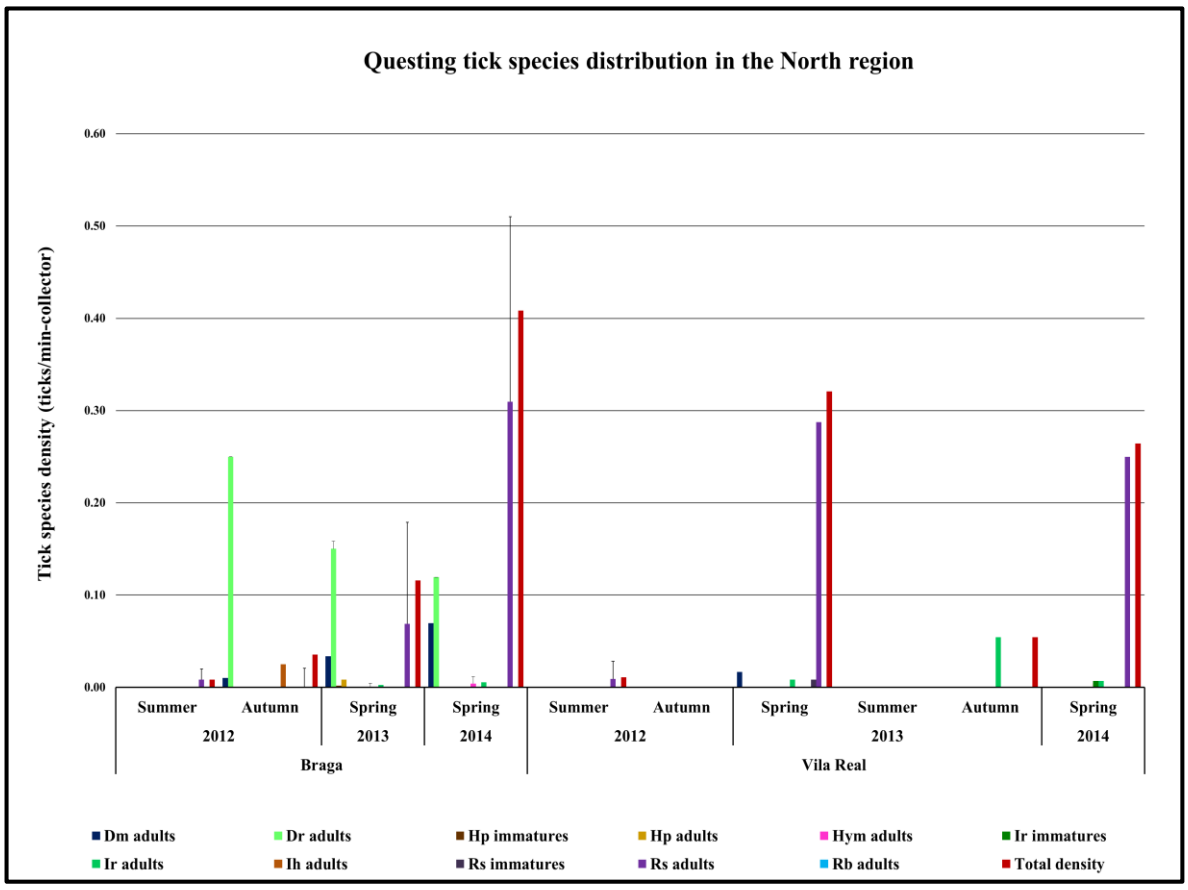

Figure 5 – Questing tick species average density and standard deviation in the North region during the two years of collections, in spring, summer and autumn seasons. (Dm – *Dermacentor marginatus*, Dr – *Dermacentor reticulatus*, Hp – *Haemaphysalis punctata*, Hym – *Hyalomma marginatum*, Ir – *Ixodes ricinus*, Ih – *Ixodes hexagonus*, Rs – *Rhipicephalus sanguineus*, Rb – *Rhipicephalus bursa*).

Questing ticks in Lisboa and Tagus Valley (LTV) region

In this region the majority of the collections were made in TNM, where *I. ricinus* immature stages were the most collected species and stage in the Springs of 2012, 2013 and 2014, while in summer immature stages of *H. punctata* and *R. sanguineus* where obtained (Figure 6). Adult ticks from *I. ricinus*, *H. punctata* and *Hy. lusitanicum* were also collected but with a low density during the Autumn of each year. In TNM during the coldest days of Autumn *H. inermis*, also known as the winter tick, was collected. Curiously, no adult *R. sanguineus* was ever collected from the vegetation in this site, although the immature stages were present.

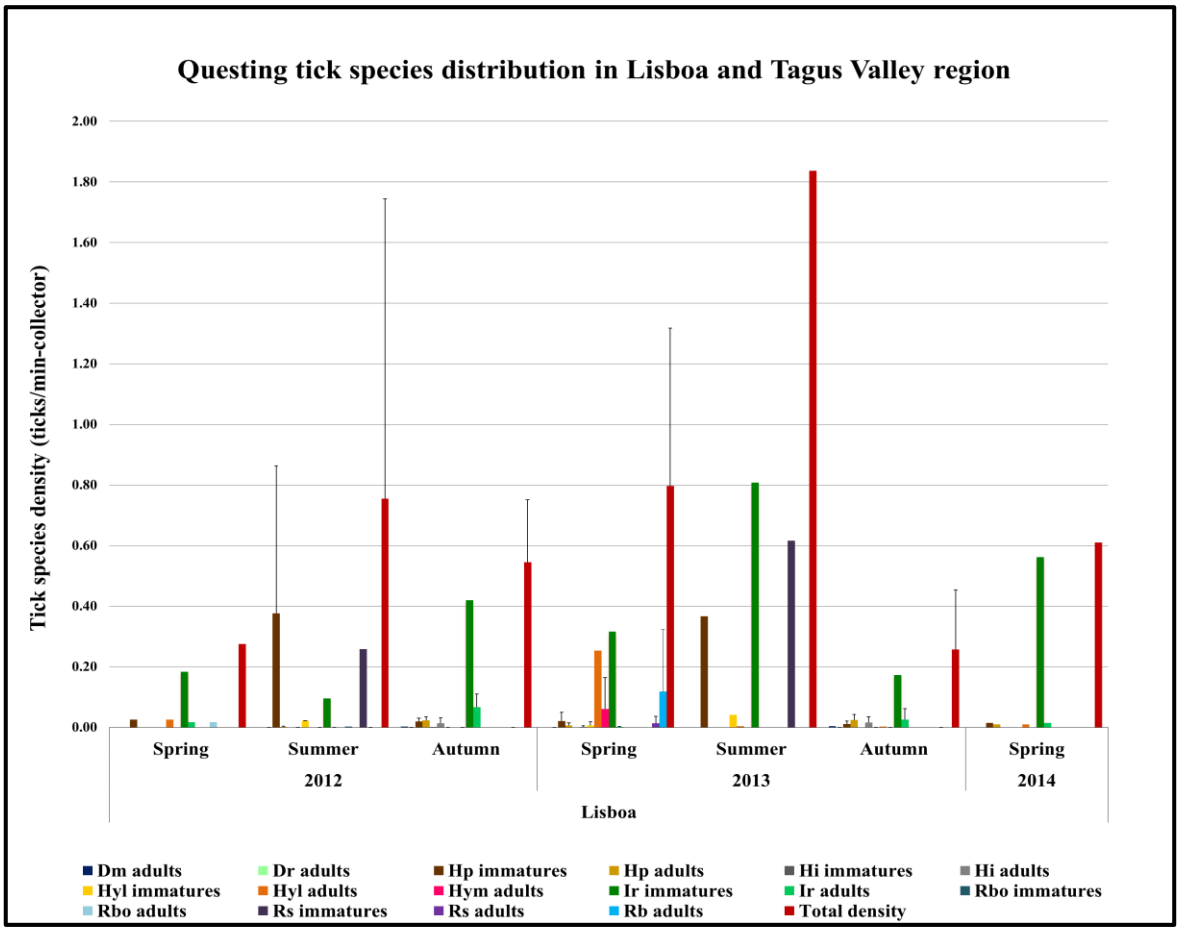

Figure 6 – Questing tick species average density and standard deviation in Lisboa and Tagus Valley region during the two years of collections, in spring, summer and autumn seasons. (Dm – *Dermacentor marginatus*, Dr – *Dermacentor reticulatus*, Hp – *Haemaphysalis punctata*, Hi – *Haemaphysalis inermis*, Hyl – *Hyalomma lusitanicum*, Hym – *Hyalomma marginatum*, Ir – *Ixodes ricinus*, Rbo – *Rhipicephalus boophilus*, Rs – *Rhipicephalus sanguineus*, Rb – *Rhipicephalus bursa*).

Questing ticks in the South region

In the South region the collections were carried out only during Spring (Figure 7), although a collection was made in the Setúbal district in the Summer of 2013, but no ticks were collected, since the land had been plowed. Regarding tick species *R. sanguineus* was the most abundant species in the three districts, although *I. ricinus* and *D. marginatus* were also collected but with a lower density, particularly in one of the collection sites in Setúbal district which was a hunting area with wild boars.

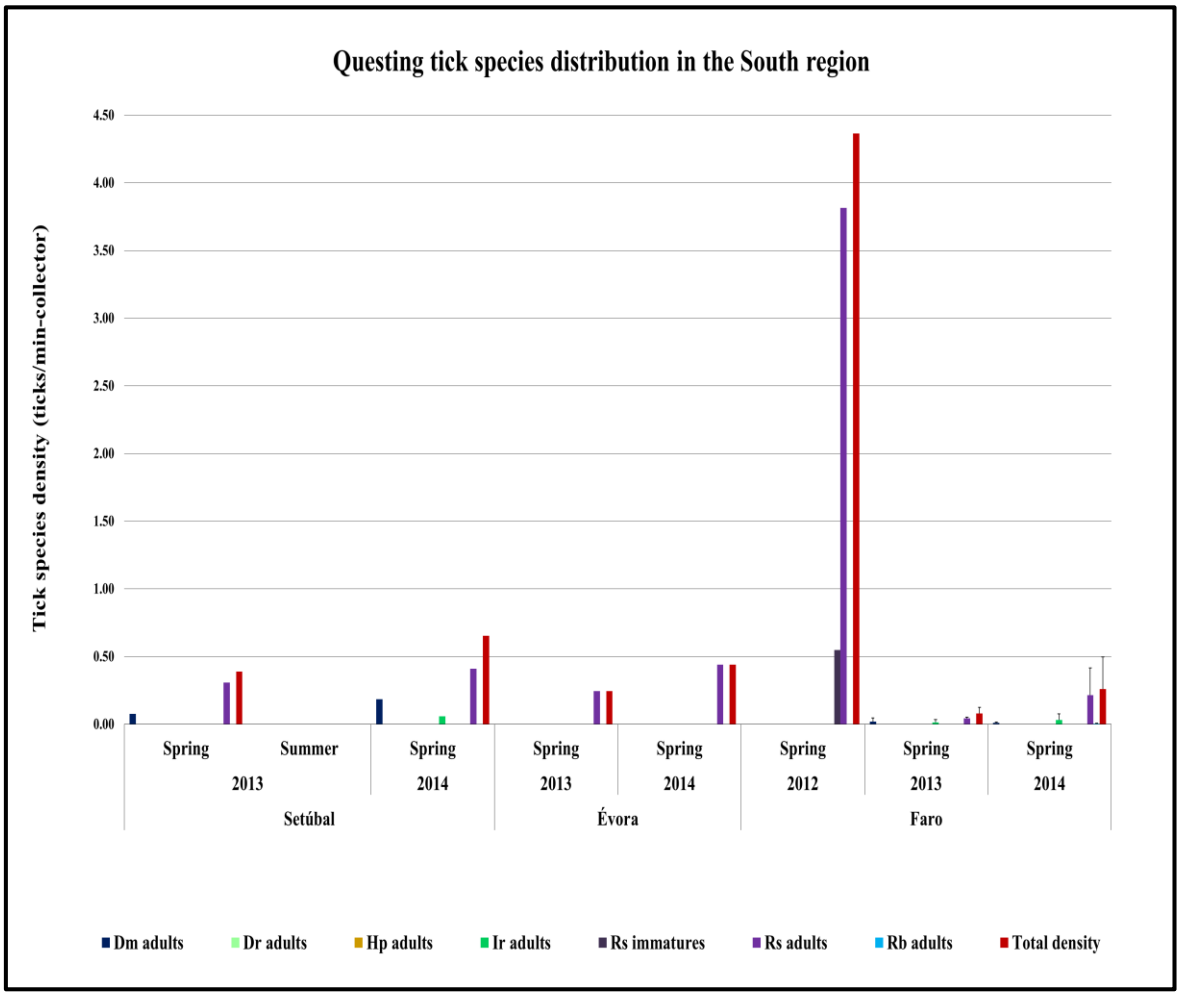

Figure 7 – Questing tick species average density and standard deviation in South region during the two years of collections, in spring and summer seasons. (Dm – *Dermacentor marginatus*, Dr – *Dermacentor reticulatus*, Hp – *Haemaphysalis punctata*, Ir – *Ixodes ricinus*, Rs – *Rhipicephalus sanguineus*, Rb – *Rhipicephalus bursa*).

Bioecological analysis of ticks collected from the vegetation

Total tick density, and the most abundant species were analysed according to environmental variables regarding the collecting sites.

Total tick densities were significantly different according to: 1) region of the collections, among LTV and North region, being higher in LTV (KW = 20.614, $P < 0.0001$); 2) season, higher in Spring than Summer (KW = 8.716, $P = 0.01$); 3) elevation, being higher below 100m than above 200m (KW = 20.302, $P < 0.0001$); 4) the three types of vegetation although the *P* value was near 0.05 (KW = 6.047, $P < 0.049$), resulting in no differences in pairwise comparisons. No differences were obtained for the habitats.

Total tick density showed a significant negative correlation with the temperature (Spearman's rhô = -0.462, $P = 0.001$) and the distance to waterline (Spearman's rhô = -0.479, $P = 0.001$), but no correlation with relative atmospheric humidity.

Identical analysis was performed for the five more representative tick species in the three regions, namely *D. marginatus*, *H. punctata*, *Hy. lusitanicum*, *I. ricinus* and *R. sanguineus.* Concerning the regions, *H. punctata*, *Hy. lusitanicum* and *I. ricinus*, were more abundant in LTV (KW = 17.543, $P < 0.0001$), in comparison to the North and the South regions (Figure 8), while *R. sanguineus* was higher in South than LTV (KW = 14.579, $P = 0.001$). No differences were obtained for *D. marginatus* ($P > 0.05$).

Regarding the seasons, *I. ricinus* was the species with the higher differences between seasons, namely it was more abundant in autumn than in either spring or summer (KW = 11.767, $P = 0.003$), (Figure 9); *R. sanguineus* was more abundant in Spring than in autumn (KW = 22.254, $P < 0.0001$); *H. punctata* in autumn than in Spring (KW = 8.981, $P = 0.01$); and *D. marginatus* in spring than in summer (KW = 9.046, $P = 0.01$). No differences were obtained for *Hy. lusitanicum* ($P > 0.05$).

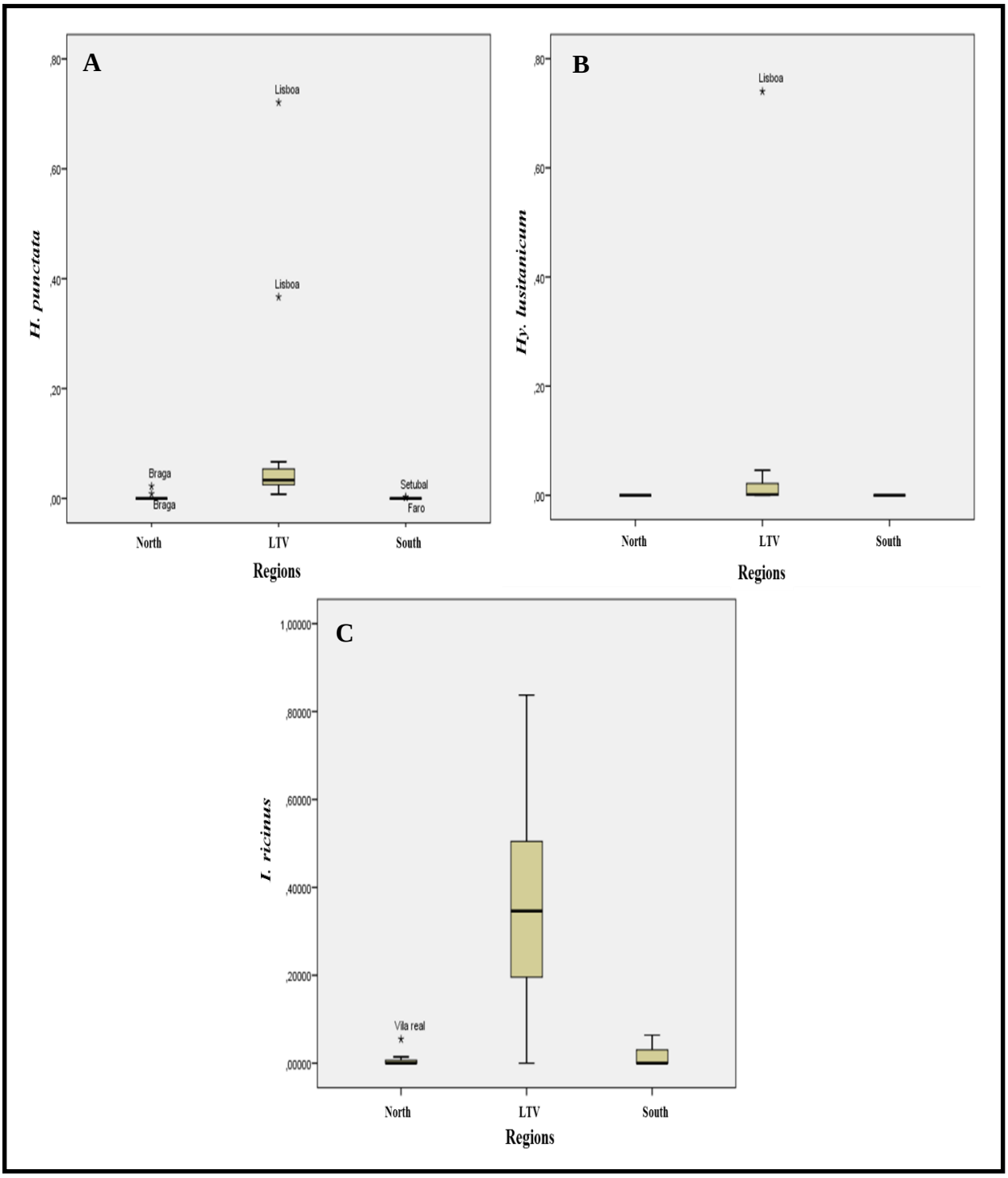

Figure 8 – Box-plot analysis depicting the distribution of (A) *H. punctata*, (B) *Hy. lusitanicum* and (C) *I. ricinus* within each region. Y axis represent ticks/min-collector.

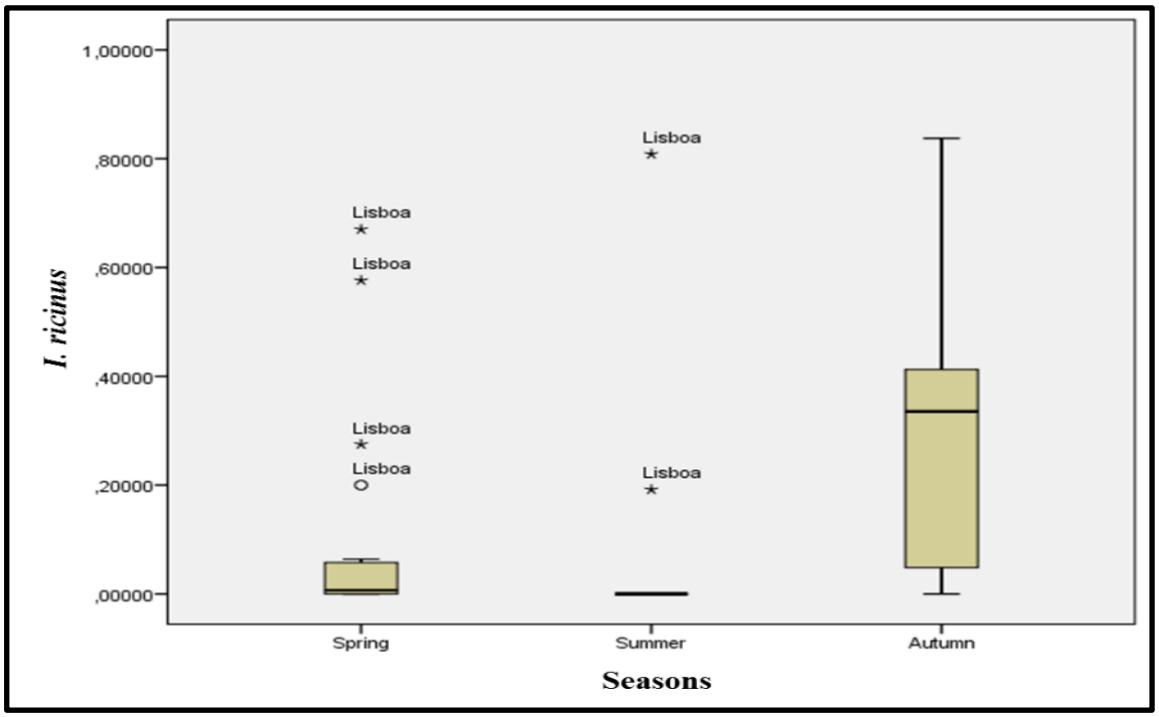

Figure 9 – Box-plot analysis depicting the densities of *I. ricinus* in each of the surveyed seasons. Y axis represent ticks/min-collector.

For the elevation *I. ricinus*, *Hy. lusitanicum* and *D. marginatus* were more abundant in elevations below 100m than in 100-200m or above 200m (KW = 11.173, *P* = 0.004), while *H. punctata* was more abudant in elevations between 100-200m (KW = 26.965, *P* < 0.0001). No differences were obtained for *R. sanguineus* (*P* > 0.05).

Concerning the vegetation only *I. ricinus* and *H. punctata* reveled to be more abundant in shrubland vegetation than in pasture and forest vegetations (KW = 15.643, *P* < 0.0001). In relastionship to the habitat, *I. ricinus* was more abundant in hunting & forest areas than in pasture & agriculture and peri-urban & public parks (KW = 7.000, *P* = 0.03); and for *H. punctata*, differences were obtained between the habitats (KW = 7.858, *P* = 0.02), however, the pairwise comparisions showed the same distribution in the three habitats.

Finally *H. puntacta* and *I. ricinus* had a negative correlation with the temperature (Spearman's rhô = -0.327, *P* = 0.02; Spearman's's rhô = -0.475, *P* = 0.001, respectively) and

the distance to the waterline (Spearman's rhô = -0.629, $P < 0.0001$; Spearman's rhô = -0.562, $P < 0.0001$), although *I. ricinus* presented a positive correlation with the humidity (Spearman's rhô = 0.334, $P = 0.02$), and *Hy. lusitanicum* showed a negative correlation with the distance to waterline (Spearman's's rhô = -0.343, $P = 0.02$).

Ticks collected from hosts

A total of 2171 ticks were removed from seven different host species (n=112), distributed along seven districts from North to South Portugal, during the two-year period. Although the majority of ticks were collected from sylvatic hosts, namely cervids, the "pets" hosts were the most surveyed, especially dogs. Tick distribution according to stage was: 1 larvae (0.04%), 36 nymphs (1.7%), 1256 females (57.9%) and 878 males (40.4%), (Table 3).

Five tick genera were identified, including nine species, being *R. sanguineus* the most collected and widespread tick (858, 39.5%), followed by *I. ricinus* (743, 34.2%), *R. bursa* (278, 12.8%), *H. punctata* (148, 6.8%), *Hy. marginatum* (85, 3.9%), *Hy. lusitanicum* (35, 1.6%), *D. marginatus* (19, 0.9%), *I. hexagonus* (3, 0.1%) and *H. inermis* (2, 0.1%) (Table 3).

Concerning the distribution of ticks in the several districts, the majority of ticks were obtained from Lisboa district (1053, 48.5%), followed by Évora (350, 16.1%), Faro (341, 15.7%), Setúbal (213, 9.8%), Vila Real (115, 5.3%), Guarda (65, 3%) and Santarém (34, 1.6%), (Table 3).

Densities of the several tick species collected in each year, district and season are represented in Figure 10, being *R. sanguineus* the most abundant species collected in all districts, except in Lisboa, which was *I. ricinus* species. For a more accurate analysis the distribution of tick species was analyzed by regions, being the graphics in different scales for a better comprehension of tick densities.

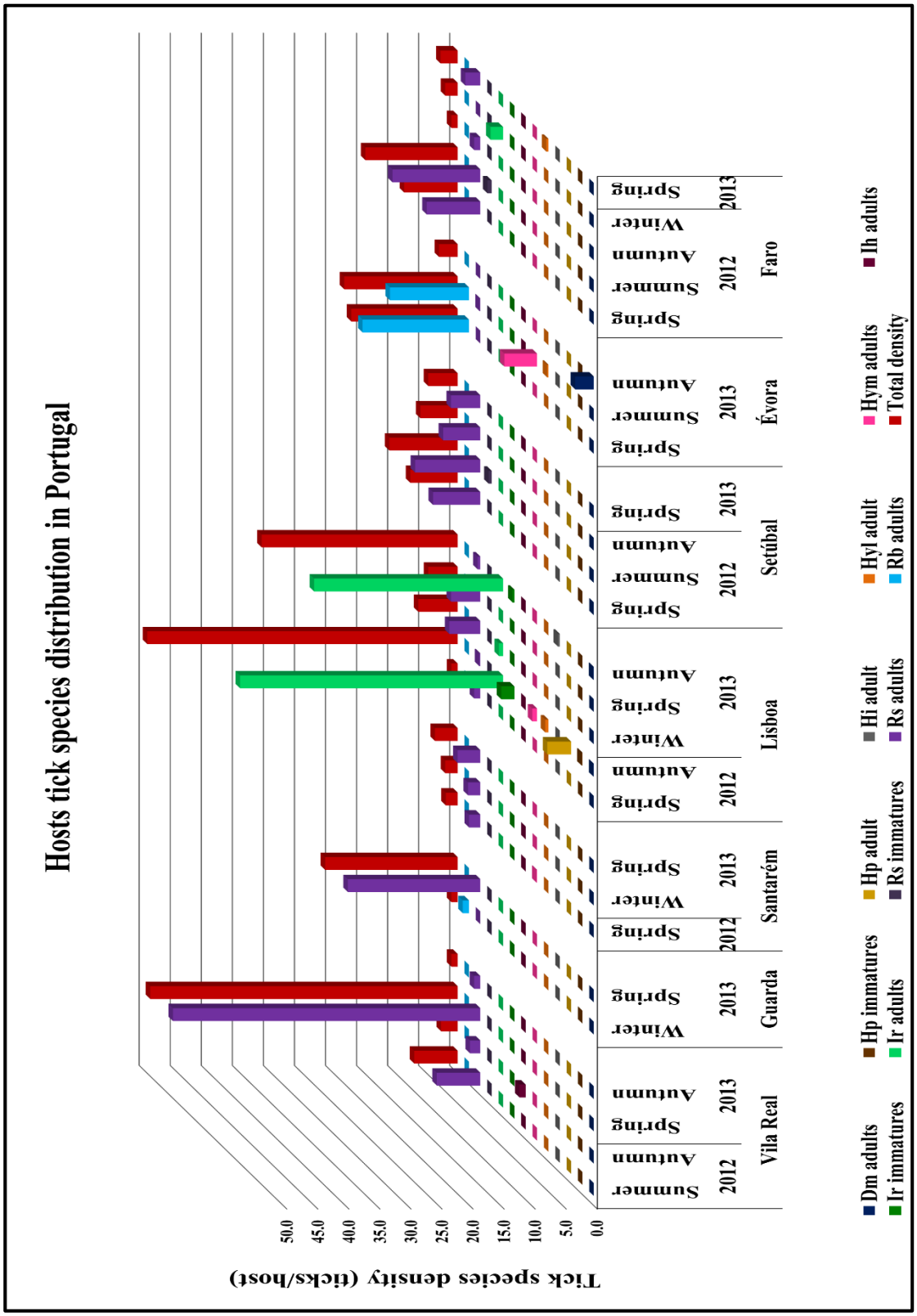

Figure 10 – Tick average density per host, for each collected species by season, year and district. Dm – *Dermacentor marginatus*, Hp – *Haemaphysalis punctata*, Hi – *Haemaphysalis inermis*, Hyl – *Hyalomma lusitanicum*, Hym – *Hyalomma marginatum*, Ir – *Ixodes ricinus*, Ih – *Ixodes hexagonus*, Rbo – *Rhipicephalus boophilus*, Rs – *Rhipicephalus sanguineus*, Rb – *Rhipicephalus bursa*.

Table 3 – Total ticks collected from hosts, per tick species and stage, collected in each district.

		Ticks collected from hosts				
		Stage				
Districts	**Tick species**	**Larvae**	**Nymphs**	**Females**	**Males**	**TOTAL**
Vila Real	*Ixodes hexagonus*		1	2		3
	Rhipicephalus sanguineus			52	60	112
	Total		**1**	**54**	**60**	**115**
Guarda	*Rhipicephalus bursa*			1		1
	Rhipicephalus sanguineus			38	26	64
	Total			**39**	**26**	**65**
Santarém	*Hyalomma marginatum*				1	1
	Rhipicephalus sanguineus			19	14	33
	Total			**19**	**15**	**34**
Lisboa	*Dermacentor marginatus*				7	7
	Haemaphysalis inermis			1	1	2
	Haemaphysalis punctata		3	69	76	148
	Hyalomma lusitanicum			30	3	33
	Hyalomma marginatum			2	24	26
	Ixodes ricinus		29	463	249	741
	Rhipicephalus sanguineus			59	37	96
	Total		**32**	**624**	**397**	**1053**
Setúbal	*Rhipicephalus sanguineus*		1	129	83	213
	Total		**1**	**129**	**83**	**213**
Évora	*Dermacentor marginatus*			7	5	12
	Hyalomma lusitanicum				2	2
	Hyalomma marginatum			8	50	58
	Rhipicephalus bursa			102	175	277
	Rhipicephalus sanguineus				1	1
	Total		**0**	**117**	**233**	**350**
Faro	*Ixodes ricinus*			1	1	2
	Rhipicephalus sanguineus	1	2	273	63	339
	Total	**1**	**2**	**274**	**64**	**341**
	TOTAL	**1**	**36**	**1256**	**878**	**2171**

Ticks collected from hosts in the North region

In the North region (Figure 11), livestock animals like sheep, and pets, such as dogs and cats, were the only hosts surveyed, being *R. sanguineus* the most collected tick, mainly during spring of each year.

In Guarda district *R. bursa* was possible to collect from a dog during the winter of 2013, and only in Vila Real district *I. hexagonus* was collected also from a dog during autumn of 2012, whose owner lived near the forest of the Natural Park of Alvão.

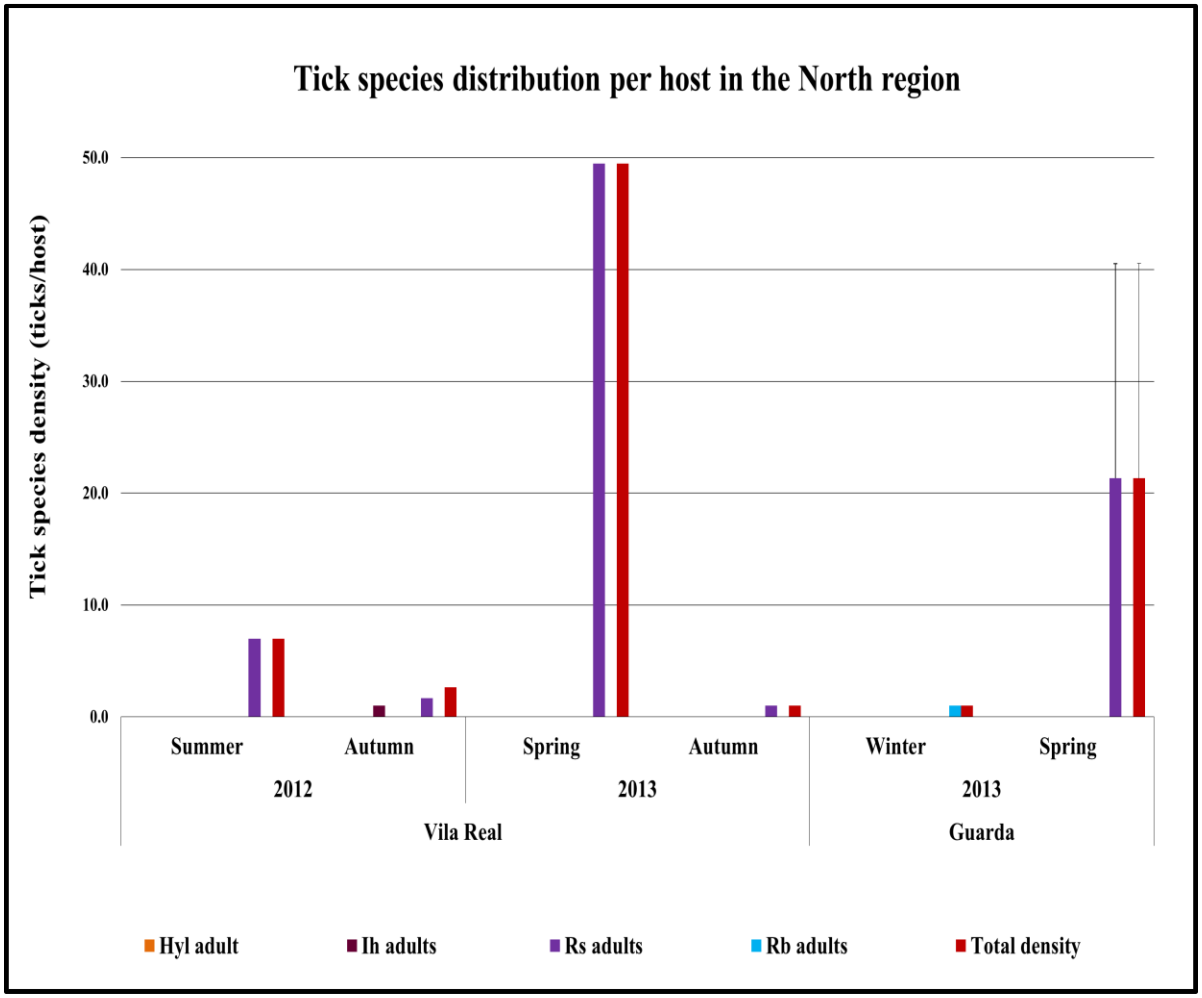

Figure 11 – Tick species average density and standard deviation per host in North region during the two years of collections, in spring, summer, autumn and winter seasons. (Hyl – *Hyalomma lusitanicum*, Ih – *Ixodes hexagonus*, Rs – *Rhipicephalus sanguineus*, Rb – *Rhipicephalus bursa*).

Ticks collected from hosts in the Lisboa and Tagus Valley region

In this region the majority of ticks were collected from sylvatic animals, namely cervids, from TNM and also from a private farm in Azambuja (Lisboa district), during the hunting season of 2012 and 2013 in autumn, being *I. ricinus* adults the most prevalent species (Figure 12). However, immature stages of *I. ricinus H. punctata* and *R. sanguineus* were also collected from these hosts. *H. inermis* was only collected in one cervid at TNM, during autumn of 2013. Curiously, in two of the survey cats, *I. ricinus* was collected, from Oeiras and Pinheiro de Loures sites, during the winter and autumn seasons of 2013, respectively.

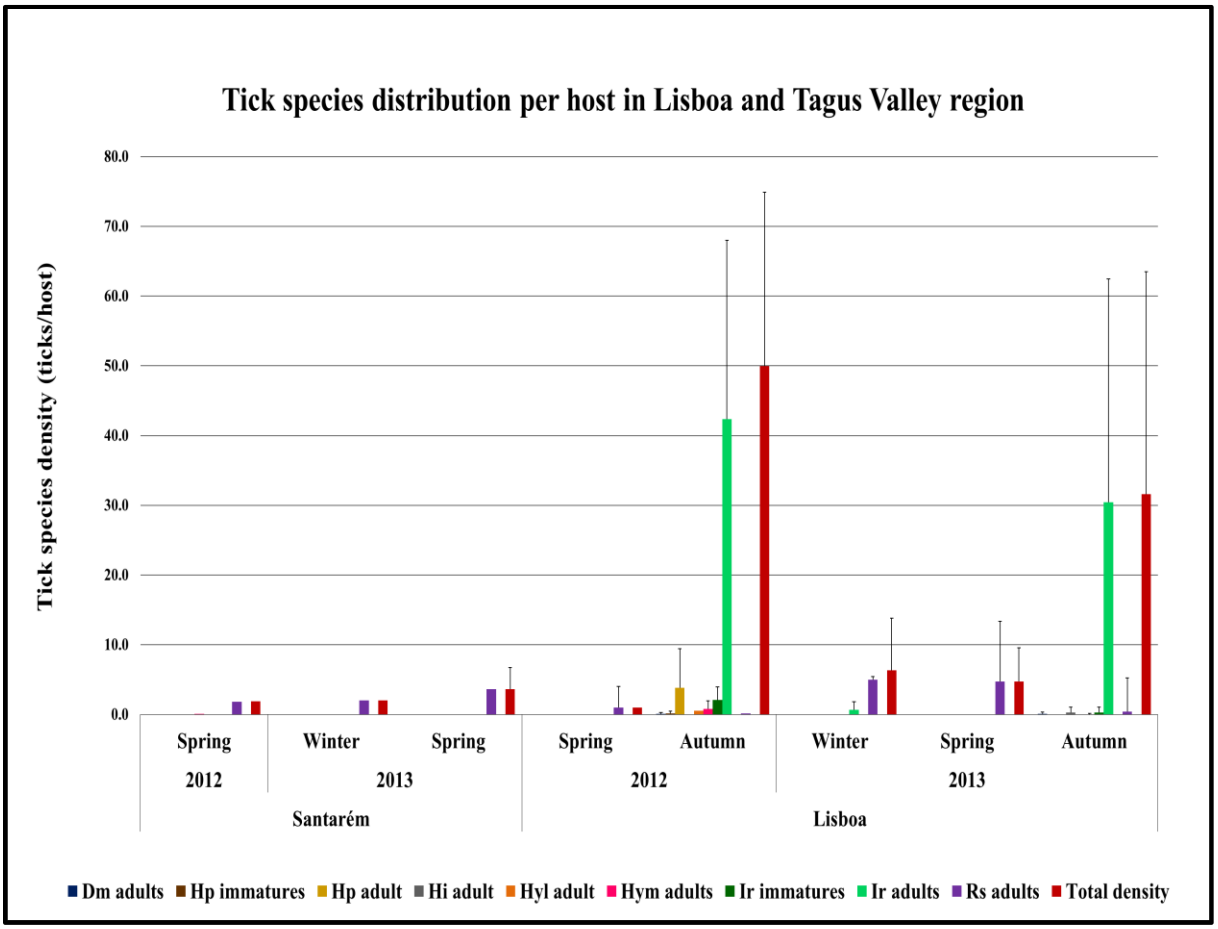

Figure 12 – Tick species average density and standard deviation per host in Lisboa and Tagus Valley region during the two years of collections, in spring, autumn and winter seasons. (Dm – *Dermacentor marginatus*, Hp – *Haemaphysalis punctata*, Hi – *Haemaphysalis inermis*, Hyl – *Hyalomma lusitanicum*, Hym – *Hyalomma marginatum*, Ir – *Ixodes ricinus*, Rs – *Rhipicephalus sanguineus*).

Hosts Tick species distribution in the South region

In the South region *R. sanguineus* was the most widespread species (Figure 13), mainly collected from dogs, the most surveyed hosts. However, during the spring and summer of 2013, *R. bursa* showed a higher density, being collected from a farmhouse that bred cattle. *D. marginatus* was also collected from one wild boar during the hunting season of 2013 autumn. Interestingly, *I. ricinus* species was obtained from a cat in Faro district during 2013 winter.

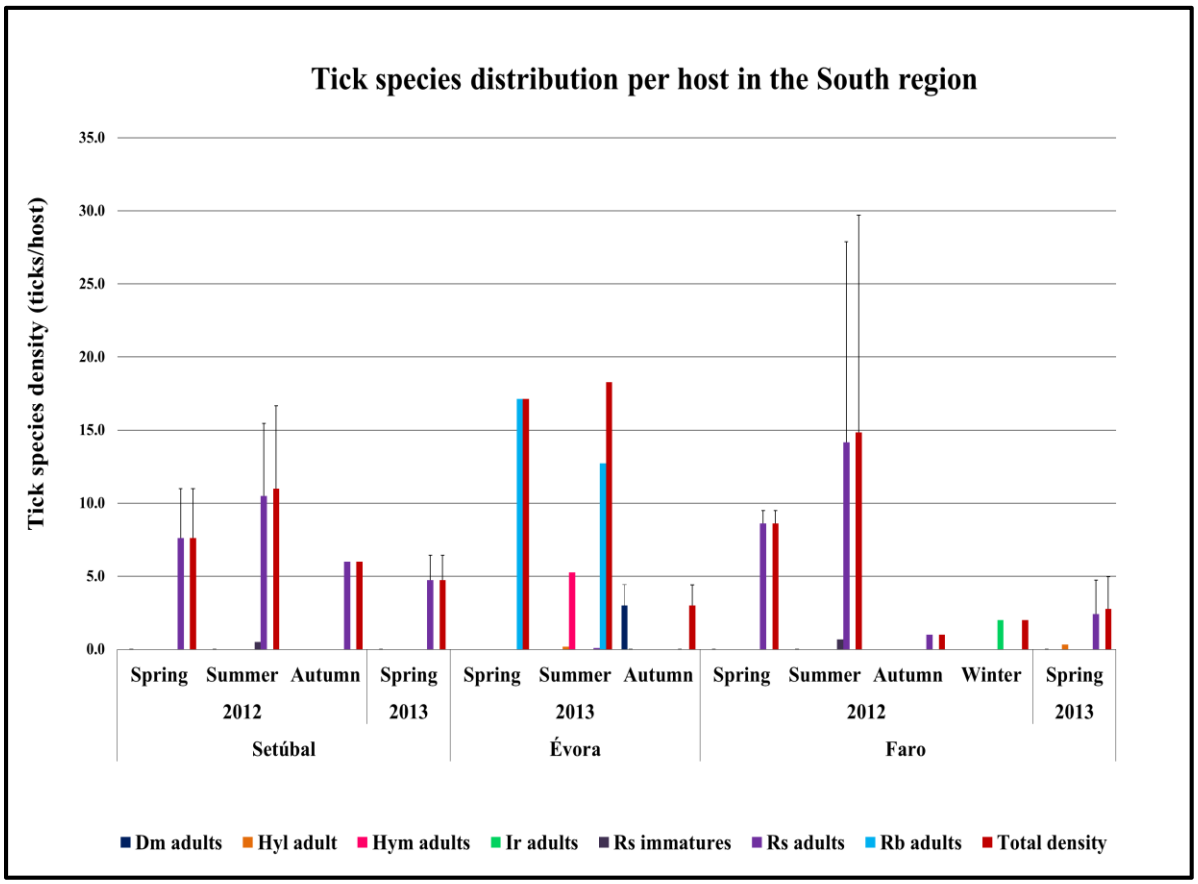

Figure 13 – Hosts tick species average density and standard deviation in South region during the two years of collections, in spring, summer, autumn and winter seasons. (Dm – *Dermacentor marginatus*, Hyl – *Hyalomma lusitanicum*, Hym – *Hyalomma marginatum*, Ir – *Ixodes ricinus*, Rs – *Rhipicephalus sanguineus*, Rb – *Rhipicephalus bursa*).

Bioecological analysis of ticks collected from hosts

Total tick density, and most abundant species were analysed according to environmental variables regarding the collecting sites.

Total tick densities were significantly different according to: 1) the season, higher in the Autumnn than in the Spring (KW = 17.221, $P = 0.01$), and in the Winter (KW = 26.462, $P = 0.04$), (Figure 14A); 2) the hosts, being more abundant in sylvatic than in pets (KW= -28.498, $P < 0.0001$) and in livestock hosts (KW = 27.821, $P = 0.02$), (Figure 14B). No differences were observed for the three regions ($P > 0.05$).

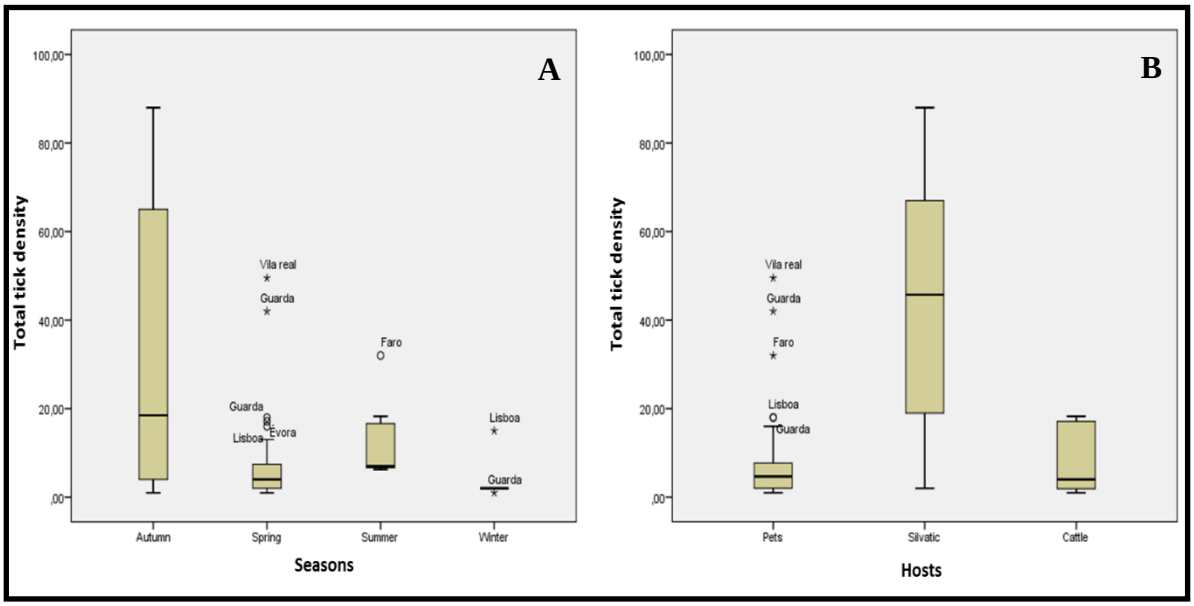

Figure 14 - Box-plot analysis depicting the distribution of total tick density in A) each season and B) per host type. Y axis represent ticks/host.

Analysis at the vegetation and the habitat level were not possible, due to the lack of information in the majority of the collections. Moreover, the total tick density showed a significant negative correlation with temperature (Spearman's's rhô = -0.374, $P = 0.001$), being the daily temperature, from stations operated close to the collections sites, obtained from "Instituto Português do Mar e da Atmosfera (IPMA)".

Identical analysis was performed for the two more representative tick species in the several hosts, namely *I. ricinus* and *R. sanguineus.* Concerning the regions, *I. ricinus*, were more abundant in LTV, in comparison to the North (KW = -15.770, *P* = 0.03) and the South (KW = 14.715, *P* = 0.001) regions (Figure 15A), while *R. sanguineus* was lower in LTV than in the North (KW= 19.701, *P* = 0.04) and in the South (KW= -14.199, *P* = 0.02) regions (Figure 15B).

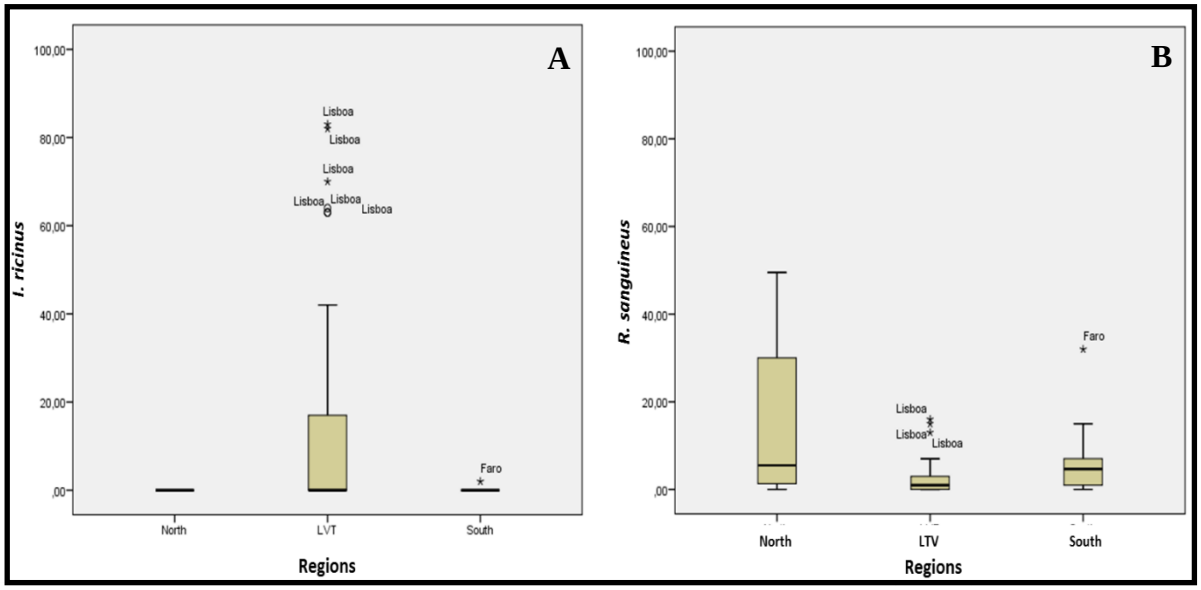

Figure 15 – Box-plot analysis demonstrating the distribution of A) *I. ricinus* and B) *R. sanguineus* within each region. Y axis represent ticks/host.

For the seasons *I. ricinus* was more abundant in Autumn than in Spring (RW = 25.227, *P* < 0.0001) and summer (KW = 25.227, *P* = 0.001), and in contrast *R. sanguineus* was less abundant in Autumn than in Spring (KW = -28,132, *P* < 0.0001) and summer (RW = -37.231, *P* < 0.0001). Concerning the hosts, *I. ricinus* was more abundant in sylvatic hosts (RW = 50.211, *P* < 0.0001), and *R. sanguineus* in pets (KW = 29.302, *P* < 0.0001). For the elevation *I. ricinus* was more abundant in elevations below 100m (MW (Mann-Whitney) = 292.500, *P* = 0.02), and no significant differences were found for *R. sanguineus*.

For the temperature and waterline only *I. ricinus* showed a significant negative correlation with the temperature (Spearman's rhô = -0.425, *P*< 0.0001).

Borrelia burgdorferi s.l. infection rate

A total of 3178 ticks from the vegetation and 1718 ticks from the hosts were analyzed for *B. burgdorferi* s.l. DNA, by the two nested-PCR targeting the *flaB* gene and the intergenic space region 5S-23S. Concerning ticks collected from the vegetation, ticks from LTV region were the most infected (7.7%), followed by South region (5.1%), and North region (1.8%), being *I. ricinus* nymphs the most infected species (Table 4).

Table 4 – *Borrelia burgdorferi* s.l. infection rate in questing ticks from the three surveyed regions (North, LTV and South).

	Vegetation								
	North			Lisboa and Tagus Valley			South		
Tick species	***B.b.*s.l. Pos**	**Total samples**	**%**	***B.b.*s.l. Pos**	**Total samples**	**%**	***B.b.*s.l. Pos**	**Total samples**	**%**
D. marginatus	1	10	10.0						
H. punctata				4	79	5.1			
Hy. lusitanicum				1	69	1.4			
I. ricinus	4	21	19.0	56	647	8.7	18	56	32.1
R. sanguineus	2	365	0.5				2	335	0.6
Total	**7**	**396**	**1.8**	**61**	**795**	**7.7**	**20**	**391**	**5.1**

*B.b.*s.l. – *Borrelia burgdorferi* sensu lato; Pos – positive; *D.* – *Dermacentor*; *H.* – *Haemaphysalis*; *Hy.* – *Hyalomma*; *I.* – *Ixodes*; *R.* – *Rhipicephalus*.

The overall infection rate for *I. ricinus* was significantly different for the three regions (χ^2=17.66, Df=2, P<0.0001), being higher in LTV region (7.7%), compared to the North region (1.8%, χ^2=17.09, Df=2, P<0.0001), and in the South region (5.1%) compared with the North region (1.8%, χ^2=6.73, Df=2, P<0.0001), but not significantly different between LTV and South regions.

For ticks collected from hosts (Table 5), only five ticks were positive for *B. burgdorferi* s.l. DNA, namely four from LTV region (two *I. ricinus* (0.5%), one *H. punctata* (1.1%), and one *R. sanguineus* (0.4%)); and one from the South region (*R. sanguineus* (0.2%)). No positive

samples were obtained in the North region. Due to the low infection rate, no statistical analysis was performed.

Table 5 - *Borrelia burgdorferi* s.l. infection rate in ticks collected from hosts in the two surveyed regions (LTV and South).

	Hosts					
	Lisboa and Tagus Valley			South		
Tick species	***B.b.*s.l. Pos**	**Total samples**	**%**	***B.b.*s.l. Pos**	**Total samples**	**%**
H. punctata	1	87	1.1			
I. ricinus	2	474	0.4			
R. sanguineus	1	120	0.8	1	543	0.2
Total	**4**	**681**	**0.6**	**1**	**543**	**0.2**

*B.b.*s.l. – *Borrelia burgdorferi* sensu lato; Pos – positive; *H.* – *Haemaphysalis*; *I.* – *Ixodes*; *R.* – *Rhipicephalus*.

Discussion

Portugal presents favorable climate conditions and suitable hosts that benefit tick abundance and distribution, and maintenance of tick-borne diseases in nature. The particularity of sharing both dominant Atlantic and Mediterranean vegetation and climatic features in the North-Northwest and Southern parts respectively, is peculiar to this country, among few others such as France and Spain.

In the present study, we detail the distribution of Ixodidae species from the vegetation and hosts, in nine districts of mainland Portugal, where the presence of *I. ricinus* species was previously reported (Baptista et al., 2004; Estrada-Peña & Santos-Silva, 2005; Baptista, 2006; CEVDI, 2013, 2014). The most widespread tick species at the vegetation level was *R. sanguineus*, present in all sampled sites with the highest density, except in LTV region, where *I. ricinus* were the most prevalent. This fact, can be related with the collection site in LTV region that was mainly TNM. This natural reserve presents unique edapho-climatic characteristics concerning Fauna and Flora which allows the occurrence of the complete life cycle and abundance of ixodids.

Concerning the ticks collected from the hosts, *R. sanguineus* was also the most abundant species, except in Évora district where *R. bursa* presented the higher density and Lisboa with *I. ricinus* as the most prevalent species. Moreover, and accordantly with previous studies (Santos-Silva et al., 2011), females, rather than males or immature stages, were the stage most found attached to the hosts, indicating a more zoophilic behavior by this gender.

In addition, dogs were the most surveyed hosts, and *R. sanguineus* are still regarded as the main tick species found in this animal species (Santos-Silva et al., 2006a; Santos-Silva et al., 2011; Maia et al., 2014), although, in the North of the country some dogs were found to be parasitized with *I. hexagonus* which is more frequent to occur in dogs from Northern Europe (Ogden et al., 2000; Foldvari & Farkas, 2005). However, the higher tick density of hosts was obtained from sylvatic hosts, namely cervids, which were parasitized by several tick species being the most prevalent species *I. ricinus* (76%) and *H. punctata* (16%).

In this study *R. turanicus* was not collected, although it has been previously reported in mainland Portugal (Papadopoulos et al., 1992; Dias et al., 1994; Caeiro, 1999; Silva et al., 2001; Estrada-Pena et al., 2004; Santos-Silva et al. 2006a; Santos-Silva et al., 2006b; Formosinho et al., 2006; Rosalino et al., 2007), its presence on the Mediterranean area is questionable, according with Walker and collaborators (2000). Also, molecular data analysis from Santos-Silva and collaborators (2011), proved that the ticks commonly called *R. turanicus* in Portugal by morphological analysis, are genetically indistinguishable from *R. sanguineus*, pointing towards to the occurrence of a single species in Portugal, *R. sanguineus*, characterized by a high level of morphological polymorphism (Santos-Silva et al., 2011).

Regarding each tick species collected from the vegetation and from the hosts in each district: *Dermacentor marginatus*, known as the ornate sheep tick, can be found up to 800-1000 m of elevation (Estrada-Peña & Santos-Silva, 2005), in temperate and dry climate regions, supporting large oscillations of temperature, with the capacity to colonize several types of vegetation. This species is considered Mediterranean (Rubel et al., 2016), being reported in several countries from Europe, Northern Africa and Asia. In our study only adult forms of *D. marginatus* were collected during Spring and Autumn, from the vegetation at Braga, Vila Real, Lisboa, Setúbal and Faro districts. Curiously, this species was more abundant in lower

elevations (below 100m) than in the higher elevations, probably due to the lack of hosts in those. Regarding hosts, this tick species was only present in livestock (cattle) and sylvatic (wild boar) hosts, at Lisboa and Évora districts. The absence of immature stages is because the small hosts like the wood mouse, a usual host for these stages, were not surveyed in this study. Similar results were also obtained by previous studies (Baptista, 2006; Santos-Silva et al., 2011; CEVDI, 2014).

Dermacentor reticulatus is the second most reported tick species in Central Europe after *I. ricinus* (Rubel et al., 2014). It has a broad geographical overlap with *D. marginatus* from Northern Portugal through Kazakhstan and western Siberia (Rar et al., 2005), but generally extends more to the North than *D. marginatus*. In more humid habitats *D. reticulatus* often occurs sympatrically with *I. ricinus* (Rubel et al., 2016). In Portugal it was previously found in eight of central-Northern regions, namely Bragança, Porto, Vila Real, Viseu, Aveiro, Castelo Branco, Leiria and Lisboa (Santos-Silva et al., 2011). In the present study, this species was only found in vegetation at Braga district, in a site very close to Vila Real district frontier, during spring. No previous studies had revealed the presence of *D. reticulatus* in Braga district, being the extension of this species to the neighbor district probably associated with host expansion, climatic changes or modifications in land usage (Bullová et al., 2009). No immature stages were collected since this stages usually feed in rodents, that were not surveyed in our study.

Haemaphysalis inermis normally prefers biotopes where cervids occur in different types of forest. As adults they are found on cervids during winter time (November to April) which led to its trivial name, winter ticks. Their distribution region is stretched from France via countries of the Balkans until Iran and South Asia (Mehlhorn, 2015). In our study, *H. inermis* was only collected at Lisboa district, namely in TNM and only during the cold days of autumn (October, November), no collections were made in the North or the South regions during these days due to logistical problems. Adults and one larvae were collected from the vegetation, and also two adults from a cervid, during the hunting season. The occurrence of immature forms in the vegetation is very sporadic, since this stages stays attached to the host for a rather short period and fall quickly down to soil, where the molting period needs often

up to one year (Santos-Silva et al., 2011; Burger et al., 2013; Mehlhorn, 2015). The small number of specimens collected is not unusual, and is probably due to the very small oviposition, although this species reached several Central European countries, the Middle East and South Asia, the number of collected ticks was always low. However, in the last decades due to ecological and climatic changes, and also a higher host availability, *H. inermis* species increased its abundance and occurrence in some countries like Hungary (Hornok & Farkas, 2009) and Spain (Barandika et al., 2008, Portillo et al., 2008). Therefore in the future, an expansion of *H. inermis* distribution might be observed in other cold and humid regions of Portugal.

Regarding *H. punctata,* they occur in different habitats reaching from humid regions with numerous plants until semiarid regions in Central Asia and North Africa. In our study *H. punctata* (one nymph and five adults) was, to the best of our knowledge, collected from the vegetation at Braga district, for the first time, since there are no information reporting the presence of this species in this district. Moreover, adults of *H. punctata* were also collected at Lisboa, Setúbal and Faro districts with low densities, and the immature stages were only present at TNM in Lisboa, mainly larvae during summer, corroborating previous studies (Baptista, 2006). Concerning the hosts, this species was only collected from cervids in TNM in Lisboa district, during the hunting season. The low number of ticks in Southern areas of Portugal was also observed by Estrada-Peña & Santos-Silva (2005), in a study based on collections from ruminants. This fact can further be related with the high temperatures registered during the collections, as most took place in late spring and summer. Moreover, this species showed a negative correlation with temperature.

Hyalomma lusitanicum is well adapted to meso Mediterranean climate summer conditions unlike *I. ricinus* (Li et al, 2012; Estrada-Peña & Estrada-Sánchez, 2014,), and is usually restricted to the Mediterranean region in the South of Europe and North Africa. In Portugal it has been reported mainly in the South of the country (CEVDI, 2014), and in our study it was collected at Lisboa, Évora and Faro districts, being collected from the vegetation only at Lisboa, and from the hosts (livestock and sylvatic) in the three districts. The immature stages were only found in TNM in Lisboa, mainly during summer but without significant difference.

Interestingly, this species was also found parasiting one of the collectors, although parasitism of humans is unusual, reflecting the availability of hosts in space and time for a tick to feed, not necessarily revealing its particular preference, although this species is not considered to be anthropophilic (Toledo et al., 2009). However, in Portugal it has been frequently found biting humans (Santos-Silva et al., 2011). Regarding the parasitism of sylvatic hosts, like cervids, this is considered accidental, and was likely favored by the availability of cervids in a fenced area (TNM).

Hyalomma marginatum is present in Africa, Europe and Asia, normally associated with livestock production, birds and accidentally man. In our study it was found in the vegetation at Braga and Lisboa districts, corroborating previous studies (Baptista et al., 2004), and in the hosts namely livestock and sylvatic hosts at Santarem, Lisboa, Évora and Faro districts. No immature stages were found, probably due to the fact that this stages are usually found in birds (Estrada-Peña et al., 2004; Apanaskevich & Horak, 2008), that were not possible to survey in our study.

Questing *I. ricinus* species showed a widespread distribution, being present in six of the nine survey districts, namely Braga, Vila Real, Aveiro, Lisboa, Setúbal and Faro. This results are in agreement with the previous ones from CEVDI reports of 2013, 2014 and 2015, and Baptista and collaborators (2004). The district with more abundance of *I. ricinus* species and where all stages were present, was Lisboa namely in TNM site (95%), due to the favorable conditions regarding the habitat, climate, vegetation and potential hosts. This area has a unique climate, presenting high humidity during most of the year, corroborating our results concerning the positive correlation between *I. ricinus* and the humidity. Regarding the hosts, *I. ricinus* tick was found in Lisboa district parasiting pets and sylvatic hosts (cervids), and in Faro district parasiting pets (cats). Only adult and nymph stages were present in the hosts, being the nymphs collected from the cervids. These results are consistent with other studies from Europe (Beichel et al., 1996; Ogden et al., 2000; Nijhof et al., 2007; Smith et al., 2011; Eichenberger et al., 2015; Otranto et al., 2015).

Rhipicephalus bursa is mainly restricted to the Mediterranean portions of Europe and Africa (Estrada-Peña & Santos-Silva, 2005). It is a thermophilic species associated with areas of

open vegetation, being able to survive in areas with large oscillations of temperatures towards extreme maximum (Papadopoulos et al., 1996). Although in Portugal this species has been found in the Northern areas associated with coldest zones. In our study only *R. bursa* adult forms were collected, from the vegetation at Vila Real and Lisboa districts, and from the hosts (cattle) at Évora and Faro districts, corroborating previous results from Santos-Silva and collaborators (2011).

Rhipicephalus sanguineus present a wide world distribution, being the most abundant species in Portugal (Santos-Silva et al., 2011). This species is well adapted to all environments from the ecological point of view, tolerating high variations of temperature and relative humidity, and adjusted to a wide variation of vertebrate hosts, parasiting numerous species of sylvatic animals, and several pets species, being particularly associated to the dog and occasionally humans. In our study, this species was collected from the vegetation at Braga, Vila Real, Aveiro, Lisboa, Setúbal, Évora, and Faro districts, being more abundant in the South during spring. Moreover, this species was also collected from hosts (pets, sylvatic and livestock hosts) at Vila Real, Lisboa, Setúbal Évora and Faro districts. The immature stages were only found at Lisboa and Faro districts mainly in the vegetation, except for three ticks (one larvae and two nymphs) that were parasiting a dog at Faro district, and were collected during summer season.

The results presented in the current study, concerning *B. burgdorferi* s.l. infection rate, were in conformity with previous studies indicating a countrywide distribution of *B. burgdorferi* s.l. bacteria in questing ticks (De Michelis et al., 2000; Baptista et al., 2004). Nevertheless, *B.burgdorferi* s.l. species were also found throughout Portugal, in ticks other that their "classical" known *I. ricinus* vector, like *H. punctata*, *R. sanguineus*, *D. marginatus* and *Hy. lusitanicum*. Similar results were also obtained by Baptista (2006), in which, besides *I. ricinus* species, other tick species (*Rhipicephalus* spp, *Dermacentor* spp, *Haemaphysalis* spp) were also infected by *B. burgdorferi* s.l. agents. The presence of spirochetes in these ticks does not mean that they are capable of transmitting them to new hosts. Therefore, these tick species should not be recognized as vector competent, but be called "carrier" species, until proven to be competent vectors. Examples of these "carriers" are *I. hexagonus*, *I. muris*, *I.*

triangulicеps, I. acuminatus, I. uriae, all involved in the maintenance of spirochetes among reservoirs hosts, and also *D. reticulatus, D. andersoni, D.variabilis, D. occidentalis, H. concinna* and *H. punctata*. None of these ticks are recognized as vectors of Lyme borreliosis for their inability of transstadial transmission in natural conditions, but they might be secondary vectors (Gern et al., 1991; Kahl et al., 1992; Barbour & Fish, 1993; Angelov et al., 1996; Dolan et al., 2000; Sun & Xu, 2003).

Moreover, the higher *B. burgdorferi* s.l. infection rate, was obtained in *I. ricinus* nymphs, being these results analogous to the ones obtained by Baptista (2006) and Schwarz and collaborators (2012), that performed their study in natural reserves at TNM and Germany respectively.

Concluding, in the nine surveyed districts several tick species were possible to collect either from the vegetation as from the hosts, although we could not cover all Portuguese districts, an expansion of tick species into new regions is noticed namely *D. reticulatus* and *H. punctata* in Braga district. This fact may have numerous consequences, including modifications in their ecological characteristics, impacts on the dynamic of local host populations, and also important implications when considering the tick-borne pathogens that could affect humans and animals. More studies should be performed to provide more information about the presence and distribution of hard ticks across Portugal and their infection with *B. burgdorferi* s.l. spirochetes, as well as other agents, taking into account the ecological and climate aspects, in order to improve the development of models that allow to predict tick distribution, with significant efforts for human and animal health.

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Supplementary Data 1: Field form for tick collections in the nine districts, regarding the number of the collection, the hour, local, type of vegetation, and environmental variables (temperature, humidity, precipitation, wind, visibility).

BOLETIM DE COLHEITAS DE IXODIDEOS
Mónica Nunes doutoramento

Coletor: _____

Data:_____ **Hora:** _____ **Colheita Nº:**_____

Concelho: _____ **Freguesia:** _____

Localidade: _____ **Coordenadas GPS:** _____

Temperatura: _____ ºC **Humidade relativa:** _____ %

Condições atmosféricas:

Precipitação
Nula ☐ Fraca ☐ Moderada ☐ Forte ☐

Visibilidade
Nula ☐ Fraca ☐ Moderada ☐ Boa ☐

Vento
Nulo ☐ Fraco ☐ Moderado ☐ Forte ☐

Método de Colheita:

1. Vegetação

Número de coletores 1 ☐ 2 ☐ 3 ☐ Mais de 3 ☐ Quantos?_____

Tempo de execução: _____ min

Trajeto percorrido: _____ m

Tipo de vegetação:

1 – Vegetação do tipo herbácea (pastagem). ☐

2 – Vegetação do tipo arbustiva. ☐

3 – Vegetação arborea (floresta/bosque). ☐

2. Hospedeiro

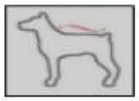
HOMEM ☐ CÃO ☐

OUTRO ☐ QUAL? _____

Número de hospedeiros pesquisados: _____

Estimativa do número de ixodídeos que parasitam o animal: 1 - 4 ☐ 11 – 20 ☐ > 50 ☐

5 – 10 ☐ 21 – 50 ☐

Local de remoção no hospedeiro: _____ 

Habitat/Utilização Humana:

1 – Pastagem / zona agrícola ☐
2 – Zona de caça ☐
3 – Limite de habitação / Jardim público ☐
4 – Outra _____ ☐

Notas:

_____

_____

_____

_____

_____

_____

_____

_____

_____

Supplementary data 2 – Identification form for the collected ticks, separated according to the collection number, origin (vegetation or host), species, stage and gender.

FICHA DE IDENTIFICAÇÃO DE IXODÍDEOS

Observador(es): _____

DADOS DA COLHEITA

Animal / solo: _____ Total de ixodídeos: _____

Data de recolha: _____ Localidade: _____

Idade do animal : Jovem ☐ Sub-adulto ☐ Adulto ☐ Sexo: M ☐ F ☐ Gestante: S ☐ N ☐

IDENTIFICAÇÃO DE IXODÍDEOS

Data de observação: _____

Género: _____ Espécie: _____

Características: _____

Número: _____

Larvas: _____ Ninfas: _____ Adultos: _____

Total Machos: _____ Total Fêmeas: _____ Fêmeas engorgitadas: _____

Género: _____ Espécie: _____

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Chapter 3

Distribution and diversity of *Borrelia* spirochetes and other tick-borne agents in questing and host-ticks

3. Distribution and diversity of *Borrelia* spirochetes and other tick-borne agents in questing and host-ticks

Globally, ticks represent one of the most significant public health risks, second only to mosquitoes as vectors for pathogen transmission. The spread and dissemination of ticks is a public health concern, especially in areas where domestic animals live in close vicinity of their owners, since they might act as reservoir hosts and direct sentinels for human infections. Any tick-borne bacterial emerging diseases such as spotted fevers, borrelioses, anaplasmoses, ehrlichioses and rickettsioses have been described worldwide. In Portugal information about the pathogenic agents circulating in vectors is limited. This chapter describes three studies regarding the molecular assessment of tick-borne pathogens from ticks collected from field vegetation or from domestic hosts, being a useful method to evaluate the risk of emerging tick-borne diseases in the studied areas.

This chapter is based on the research papers:

Nunes M, Parreira R, Maia C, Lopes N, Fingerle V, Vieira ML. 2016. Molecular identification of *Borrelia* genus in questing hard ticks from Portugal: phylogenetic characterization of two novel Relapsing Fever-like *Borrelia sp.*. *Infection, Genetics and Evolution*, 40: 266–74. doi:10.1016/j.meegid.2016.03.008;

Nunes M, Parreira R, Lopes N, Maia C, Carreira T, Sousa C, Faria S, Vieira ML. 2015. Molecular identification of *Borrelia miyamotoi* in *Ixodes ricinus* from Portugal. *Vector-Borne and Zoonotic Diseases*, 15 (8): 515-17. doi: 10.1089/vbz.2014.1765;

Ferreira A, **Nunes M**, Vieira ML, Campino L, Cardoso L. 2014. Molecular detection of bacterial and parasitic pathogens in hard ticks from Portugal. *Ticks and Tick-borne Diseases*, 5(4): 409-14. doi: 10.1016/j.ttbdis.2014.01.009.

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Research paper

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

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ABSTRACT

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

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

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

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

1. Introduction

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

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

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

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

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

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

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

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

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

2. Material and methods

2.1. Study area and tick collection

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

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

2.2. DNA extraction

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

2.3. PCR amplifications

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

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

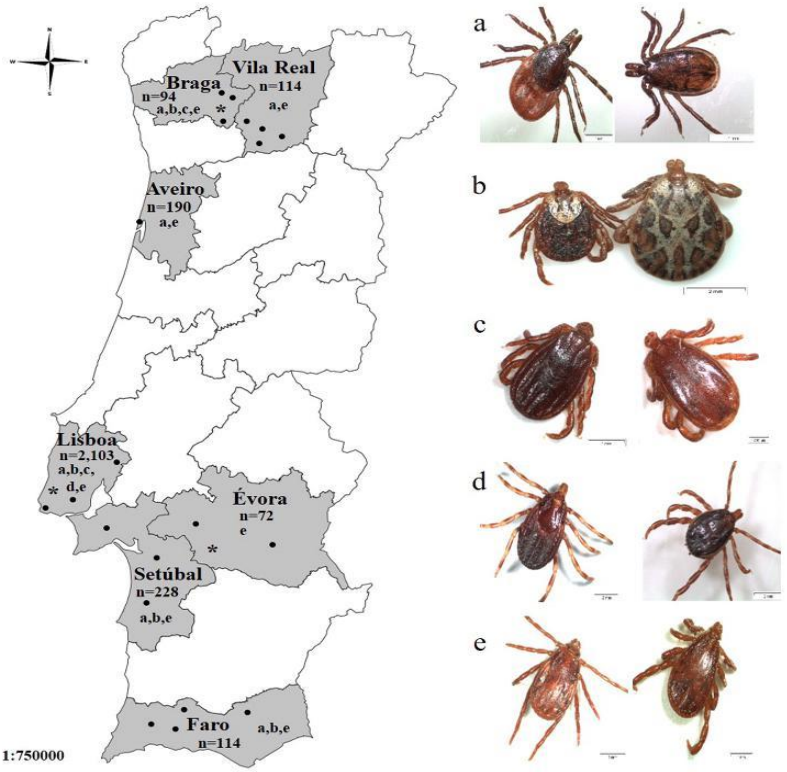

Fig. 1. Map of Portugal showing the total number of hard ticks collected by flagging per districts (Braga, Vila Real, Aveiro, Lisboa, Setúbal, Évora and Faro). Images of the different tick species collected are present (female specimens—left; male specimens—right). The black dots indicate the 23 collection sites; the genera of ticks identified in each district are indicated by letters (a — *Ixodes*; b — *Dermacentor*; c — *Haemaphysalis*; d — *Hyalomma*; e — *Rhipicephalus*); the asterisks indicate the districts were the two putative Relapsing Fever-like species were identified.

flagellin gene (*flaB*) (Wodecka et al., 2010). This included a first amplification reaction based on the use of outer primers 123f and 905r (which amplify a 774 bp DNA fragment), with a second amplification step using inner primers 220f and 824r (yielding an amplification product of 605 bp). The PCR conditions included an initial denaturation at 94 °C for 10 min, followed by 40 cycles of amplification, including denaturation at 94 °C for 30 s, annealing for 45 s at a temperature dependent on the primers used (outer primers—50 °C; inner primers—54 °C). An additional elongation step was carried out at 72 °C for 1 min with a final elongation at 72 °C for 7 min. PCR protocols were done in a separate vertical laminar flow bench using a different set of micropipettes, for PCR use-only as well filtered tips and sterilized material to ensure a contamination-free environment. *B. garinii* DNA was used as positive control and ultrapure water as negative control.

Nested-PCR products were detected by electrophoresis in 1.5% agarose gels stained with GreenSafe Premium (NZYTech), and visualized under UV light, using a Dolphin-1D Gel Image Analysis Software (Wealtec®) equipment.

2.3.2. DNA amplification from Relapsing Fever Borrelia species

For the characterization of the RF genospecies, two PCR protocols targeting the 16S rRNA, and *glpQ* genes were optimized using the primers described in Table 2. PCR conditions for the amplification of 16S rRNA (primer pair 16SB Fw/Rv) started with an initial denaturation at 94 °C for 3 min, followed by 35 cycles of amplification (denaturation at 94 °C for 30 s, annealing for 30 s at 60 °C, elongation at 72 °C for 2 min), with a final elongation at 72 °C for 7 min. Partial amplification of the *glpQ* gene was achieved using nested-PCR and a thermal profile comprising a denaturation step at 94 °C for 2 min, 40 cycles of amplification at 94 °C for 30 s, 56 °C for 1 min (outer primers: glpQ Fw1/Rv1), or 53 °C for 1 min (inner primers: glpQ Fw2/Rv2), and 72 °C for 45 s, followed by a 3 min extension phase at 72 °C. As in *B. burgdorferi* s.l. PCR protocols, negative control was prepared using ultrapure water and the same measures were taken to ensure the quality and prevent contamination of the biological material. As positive controls DNA from several RF species (*B. recurrentis*, *B. anserina*, *B. miyamotoi*, *B. parkeri*, *B. turicatae*, *B. duttoni* and *B. hispanica*), kindly provided by the German National Reference Centre for *Borrelia*, were used. PCR products were detected as described above.

2.4. DNA sequencing and analyses

The amplification products for *flaB* and IGS region were purified and directly sequenced on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, USA). The 16S rRNA and *glpQ* specific amplicons were sequenced with the oligonucleotide primers used for PCR, along with others, listed in Table 2. DNA sequences obtained in the course of this work were each assembled to generate single contigs using the CAP

Table 1
Species, stage, gender and number of collected ticks, analyzed for the presence of *B. burgdorferi* s.l. and Relapsing Fever *Borrelia* (RFB) spirochetes DNA.

		Collected and analyzed ticks					PCR-positive tick samples									
		Stage/gender					*B. burgdorferi* s.l. species					RF-like *Borrelia* sp.				
Districts	Tick species	Larva	Nymph	Female	Male	Total	Larva	Nymph	Female	Male	Total	Larvae	Nymph	Female	Male	Total
Braga	*Dermacentor marginatus*			6	4	10				1	1					
	Haemaphysalis punctata			2		2										
	Ixodes ricinus			3		3										
	Rhipicephalus sanguineus			41	38	79			1		1			1		1
Vila Real	*Ixodes ricinus*			8	7	15			2	2	4					
	Rhipicephalus sanguineus			40	59	99				1	1					
Aveiro	*Ixodes ricinus*				3	3										
	Rhipicephalus sanguineus			94	93	187										
Lisboa	*Dermacentor marginatus*			1		1										
	Haemaphysalis inermis			17	6	23										
	Haemaphysalis punctata	250	58	20	9	337	1	2		1	4	5	1			6
	Hyalomma lusitanicum		2	50	17	69		1			1					
	Hyalomma marginatum			2	16	18										
	Ixodes ricinus	440	947	32	58	1477	5	49	2		56					
	Rhipicephalus bursa			17	18	35										
	Rhipicephalus sanguineus	130	1	8	4	143										
Setúbal	*Dermacentor marginatus*			12	7	19										
	Ixodes ricinus			12	7	19			3	2	5					
	Rhipicephalus sanguineus			105	85	190										
Évora	*Rhipicephalus sanguineus*			37	35	72				2	2				1	1
Faro	*Dermacentor marginatus*			5	1	6										
	Ixodes ricinus			17	18	35			8	5	13					
	Rhipicephalus sanguineus			36	37	73										
	Total	820	1008	565	522	2915	6	52	16	14	88	5	1	1	1	8

Contig Manager tool available in BioEdit 7.0.9.0. (Hall, 1999). Nucleotide sequence (nt) similarity searches were carried out through the NCBI web server using BLASTn (megablast) and BLASTx tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The obtained data sequences were deposited in the public databases under accession numbers KR677086 to KR677091 and KT364297 (*flaB*), KT364298 to KT364305 (16S rRNA), and LC093500 to LC093507 (*glpQ*). The RFB and *B. burgdorferi* s.l. reference sequences used for the preparation of the different sequence-datasets were selected among those previously deposited in the GenBank database, on the proviso that they would be representative of (i) each of the previously described RFB and *B. burgdorferi* s.l. species with (ii) a significant sequence overlap with the sequences we have obtained, in order to maximize the number of unambiguously aligned nucleotide positions in each multiple-sequence alignment. When multiple reference sequences were available for each species, the 16S rRNA and *flaB* datasets included 1–2 sequences per species (randomly selected from those available in the databases), with the exception of (i) sequences described as *Borrelia* sp. and (ii) those more closely related to the ones obtained (e.g. *B. miyamotoi* and *B. lonestari*). In these cases, and when available, we chose to use from 3 to 5 references sequences. In the *glpQ* dataset, when available, each species was represented from 2 to 6 sequences.

Multiple alignments of nt sequences were performed using the iterative G-INS-i (*flaB* and *glpQ* sequences) or Q-INS-I algorithms (16S rRNA sequences) as implemented in MAFFT vs. 7 (Katoh and Standley, 2013). Editing of the alignments was done using the GUIDANCE guide-tree based alignment confidence (Penn et al., 2010), selecting columns with confidence levels above 0.9. For Maximum Likelihood (ML) and Bayesian phylogenetic analyses (see below), the choice of the best fitting evolutionary model was based on those defined using JModeltest2 (Darriba et al., 2012), on the basis of the AIC selection criterion. These models or the ones closest to them were used for phylogenetic reconstruction, depending on the software employed.

Phylogenetic trees were constructed using multiple approaches. Neighbor-Joining (NJ) tree reconstruction was carried out using the Mega 6.0 software (Tamura et al., 2013), and genetic distance matrixes corrected using the Tamura–Nei formula (Tamura and Nei, 1993). Mega 6.0 was also used for phylogenetic tree analysis using the ML optimization criterion. In this case the GTR + Γ (GTR—General Time Reversal; Γ—Gamma distribution) evolutionary model was chosen as being the one closest to those suggests by JModelstest (TIM3 + Γ and TPM2uf + Γ) for the analysis of *flaB*/*glpQ* and 16S rRNA sequences, respectively. Finally, a Bayesian approach to phylogenetic reconstruction was also undertaken using the GTR + Γ model and the MrBayes v3.0b4 software (Ronquist and Huelsenbeck, 2003). The analyses consisted of 5×10^7 generations starting from a random tree and four Markov chains with default heating values, sampled every 100th generation. Two separate runs were conducted for each analysis and the first 10% sampled trees discarded as burn-in. Finally, Maximum Clade Credibility trees were constructed using BEAST v1.7.5 (Drummond et al., 2012) GTR + Γ, and as coalescent priors, a Bayesian skyline plot for estimating demographic, under both strict and uncorrelated lognormal relaxed clock. These analyses were run for 1×10^8 generations starting from a random tree and sampling every 1000th generation. In each case,

Table 2
Primers used in this study for the specific analysis of Relapsing Fever *Borrelia*.

Gene	Primer designation	Sequence	Fragment size (bp)
16S rRNA	16SB Fw	5′-GAGGTGATCCAGCCACACTTTCCAG-3′	1324
	16SB Rv	5′-GCTTCGCTTGTAGATGAGTCTGCGTC-3′	
	16S Seq	5′-GAGCATACTCCCCAGGCGGCACACTTAAC-3′	
glpQ	glpQ Fw1	5′-TAGCTCAYAGRGGYGCHAGYG-3′	693
	glpQ Rv1	5′-ATCCAYGSVCCYATRCCYTC-3′	
	glpQ Fw2	5′-CCAGAACATACHYTAGARKCYAAAGC-3′	598
	glpQ Rv2	5′-TATTCATARTCYGTTGGKGMYTCDTYC-3′	

two separate runs were combined using LogCombiner (http://beast.bio.ed.ac.uk/logcombiner), and the first 10% discarded as bur-in. Convergence was monitored with Tracer v1.6 (available from http://beast/bio/ed.ac.uk/Tracer), with all ESS values were confirmed to be above 200. The phylogenetic trees were manipulated for display using Fig Tree v.1.4.2. (available at http://tree.bio.ed.ac.uk/software/figtree/). The genetic distances were calculated using the Tamura–Nei formula (Tamura and Nei, 1993), as implemented in the Mega 6.0 software (Tamura et al., 2013).

3. Results

In this study, 2915 ticks, representing both immature and adult stages, were collected across Portugal, in 23 collection sites located in seven districts. The ticks collected from each district were assorted by species based on morphological criteria, and separated by developmental stage and gender (male/female), as detailed in Table 1. Nymphs were the most prevalent development stage, followed by the larvae, and finally adults, including both females and males. The greater diversity of tick species was collected in the districts of Lisboa (the capital city), and Braga, in the north-west of Portugal. *I. ricinus* and *R. sanguineus* were the most frequently found species in these collections.

DNA of *B. burgdorferi* s.l. genospecies was detected by the two nested-PCR protocols (targeting *flaB* and IGS sequences), in 88 samples (3.3%), which included either single individuals (nymphs and adults), or pools of 10 larvae. Of these 7.3% of the total number of pools of larvae (6/82) were positive for *B. burgdorferi* s.l. DNA. On the other hand, 5.1% and 2.8%, respectively, of the total number of nymphs (52/1008), and adult specimens (30/1087), showed a positive amplification result. Sixty-three (72%) of the number of samples for which an amplicon was obtained were sequenced. The sequence data were blasted (megablast) against nucleotide references available in the GenBank/EMBL/DDBJ databases, and revealed matches with ≥97% identity with homologous sequences, all belonging to the *B. burgdorferi* s.l. complex. Fifteen (24%) of the sequences matched with *B. lusitaniae* and *B. garinii* (the most prevalent), from ticks collected at Lisboa, Setúbal, and Faro districts, followed by seven (11%) with *B. burgdorferi* s.s. from ticks collected at Vila Real, Lisboa and Setúbal districts, six (10%) with *B. valaisiana* and two (3%) with *B. afzelii*, both from ticks collected at Braga, Lisboa and Vila Real districts.

Unexpectedly, eight of the flagellin-specific amplicons revealed sequences with high identity (≥95%) with those from bacteria belonging to the RFB group. Since most of the obtained results disclosed matches to unknown species of *Borrelia* (*Borrelia* sp.), thorough genetic analyses of these strains were carried out, making use of different techniques for phylogenetic tree reconstruction.

In order to better understand the place held by the detected Portuguese *Borrelia* strains among RFB, two PCR protocols, targeting the 16S rRNA and *glpQ* genes from these bacteria, were additionally optimized, and the obtained amplification products (Fig. 2) were purified and sequenced. The determined 16S rRNA, *flaB*, and *glpQ* sequences were aligned with homologous sequences from a broad range of *Borrelia* species, including both RFB and *B. burgdorferi* s.l. downloaded from the public databases, and their phylogenetic relationships were inferred using complementary tree building methods (see Material and methods). Regardless of the analytic approach used, congruent trees

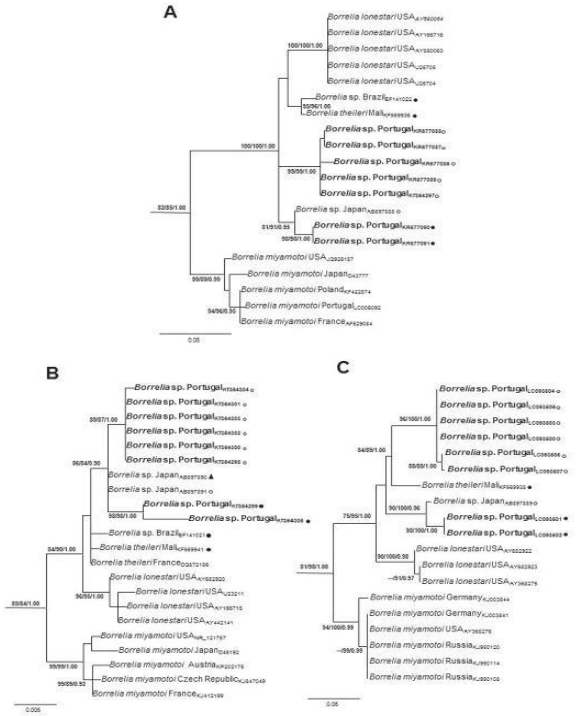

Fig. 3. Phylogenetic analysis of Relapsing Fever *Borrelia flaB* (A), 16S rRNA (B), and *glpQ* (C) sequences. At specific branch nodes bootstrap values (b) ≥75% or posterior probabilities (pp) ≥0.92 are indicated (bNJ/bML/ppBayes). Bootstrap/posterior probability values below these limits are indicated by "–". The size bar indicates the number of nucleotide substitutions per site. Open and closed circles represent, respectively, sequences amplified from *Haemaphysalis* spp. and *Rhipicephalus* spp. ticks. Triangles indicate sequences amplified from sika deer. The full-size phylogenetic trees can be found as Supplementary Fig. 1.

topologies were repeatedly obtained. As shown in Fig. 3 (and Supplementary Figs. 1, 2 and 3), the analyses of 16S rRNA, *flaB* or *glpQ* sequences showed that the majority (n = 6) of the Portuguese RFB strains (1 failed to produce a *flaB* amplicon compatible with DNA sequencing, as shown in lane 7 Fig. 2) clustered together in the RFB group, segregating away from any other reference sequences used. On the other hand, two others sequences (KT364299 and KT364305), amplified from DNA extracts of *R. sanguineus*, formed a separate cluster in all the trees. When shorter *fla*B reference sequences were analyzed (data not shown), the number of *Borrelia* sp. clustering with the two Portuguese strains increased. Curiously, these sequences of Asian (Japan) origin were either associated with *Haemaphysalis* spp. ticks or had been amplified directly from blood samples from sika deer

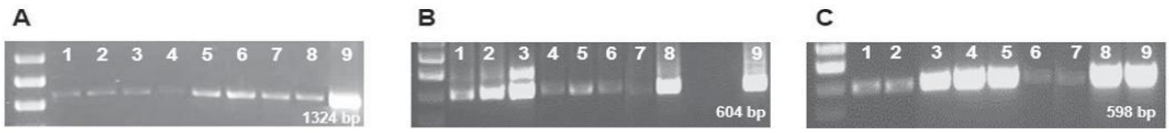

Fig. 2. Detection of RFB (Relapsing Fever *Borreliae*) DNA in extracts prepared from field-collected ticks. (A) Amplification of 16S rRNA (1324 bp amplicon), (B) *flaB* (604 bp amplicon), and (C) *glpQ* (598 bp amplicon). Lanes: 1–6: DNA amplified from *Haemaphysalis* punctata ticks; 7–8: DNA amplified from *Rhipicephalus sanguineus* ticks; 9: PCR positive control (*B. duttonii*).

(*Cervus nippon yesoensis*). In all the obtained phylogenetic trees, the unique character of the majority of the new RFB from Portugal, detect in *H. punctata* ticks, is suggested by their inclusion in a single monophyletic cluster. The position of this cluster within a larger one formed by other RFB (*Borrelia* sp., *B. lonestari* and *B. theileri*) changes slightly depending on the genetic marker used. For example, while the analysis of *glpQ* suggests they share a common ancestry with *B. theileri* (from Mali), this observation was not supported by any of the other trees. In all cases, these two clusters of Portuguese *Borrelia* sp. were reinforced by high bootstrap or posterior probability values. Despite the shared phylogenetic ancestry with both *B. theileri* and *B. lonestari*, the two clusters were clearly shown to be distinct from one-another, possibly representing two new species of RFB.

Regardless of the trees obtained (unrooted or rooted trees), and the sequence(s) eventually used as outgroup (including using *Leptospira interrogans* as outgroup in the analyses of *fla*B and 16S sequences, Supplementary Fig. 3) the topology of the trees remain essentially the same. This is especially true in what concerns the clustering of the sequences that define the RFB group, since they always form a statistically stable monophyletic cluster, where the different sequences segregate congruently in all the analyzed trees.

Additional support for the position of the strains found in our study was drawn from the calculation of genetic distances taking into consideration the two most polymorphic markers analyzed (*flaB* and *glpQ*). For both genes, the corresponding inter-group genetic distance matrixes are shown in Fig. 4. To assist the analysis, different genetic clusters defined in the *flaB* and *glpQ* trees were numbered (G1–G14), as graphically indicated in Supplementary Fig. 1.

The analysis of *flaB* distances not only clearly separated, as expected, the RFB from both the *B. turcica* (G12) and *B. burgdorferi* s.l. (G13), but also showed that some of the smallest genetic distance values were obtained upon comparisons of *B. theileri* vs. *B. lonestari* (G1 vs. G2 = 0.050). Since these are well established *Borrelia* species, it is worth mentioning that their *flaB* sequences were separated by a genetic distance value very similar to that calculated when the two clusters of Portuguese *Borrelia flaB* sequences were compared (G3 vs. G4 = 0.043). Similar observations when made upon analysis of *glpQ*. For this gene, the genetic distances between *B. hermsii* and *B. parkeri* (G9 vs G10 = 0.027) or *B. parkeri* and *B. persica* (G9 vs G11 = 0.044) were found to be smaller than those calculated when either the two groups of Portuguese *Borrelia* sequences were compared (G1 vs. G3 = 0.065), or when the former were compared with *B. theileri* (G1 vs. G2 = 0.090; G3 vs. G2 = 0.084). Therefore, both the topology of the phylogenetic trees and the analysis of genetic distances were compatible with the suggestion that the *Borrelia* detected in the course of our work may correspond to two putative RFB species.

4. Discussion

The number of publications in the literature describing *B. burgdorferi* s.l. bacteria, their distribution and association with human disease, is much larger compared to RFB. In Portugal, for example, several species of *B. burgdorferi* s.l., including *B. garinii*, *B. afzelii*, *B. burgdorferi* s.s., *B. valaisiana* and *B. lusitaniae*, have been detected over the years in *I. ricinus* as well as in other tick species (De Michelis et al., 2000; Baptista et al., 2004; Dietrich et al., 2010; Milhano et al., 2010; Norte et al., 2012; Maia et al., 2014). Furthermore, human cases with clinical symptoms compatible with LD were first identified in 1989 (Morais et al., 1989), and since then its number has increased (laboratory data not published). Nevertheless, it can be assumed that LD is underdiagnosed and underreported in Portugal. In sharp contrast, the slow pace at which the body of knowledge on RFB, transmitted by hard ticks, has built up is due, in part, to difficulties associated with their isolation under laboratory conditions using conventional cultivation techniques. As an example, regardless of being relatively well known, *B. theileri* has never been successfully cultivated in vitro despite multiple attempts, even when many spirochetes were found in tick tissues, as demonstrated by microscopic examination of Giemsa-stained tick organs (Smith et al., 1978). In any case, while the isolation of these bacteria may not be achievable on a routine basis, they are

***flaB* genetic distances analysis**

		G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13
G1	*Borrelia lonestari*													
G2	*Borrelia* sp. Br + *Borrelia theileri*	0.050												
G3	*Borrelia* sp. Portugal ○	0.057	0.048											
G4	*Borrelia* sp. Japan + *Borrelia* sp.Portugal ●	0.050	0.041	0.043										
G5	*Borrelia miyamotoi*	0.110	0.099	0.111	0.104									
G6	*Borrelia hermsii*	0.147	0.129	0.140	0.129	0.116								
G7	*Borrelia anserina*	0.156	0.135	0.144	0.145	0.104	0.128							
G8	*Borrelia coriaceae*	0.151	0.127	0.151	0.146	0.094	0.111	0.084						
G9	*Borrelia persica*	0.146	0.129	0.145	0.143	0.112	0.117	0.091	0.091					
G10	*Borrelia hisp+mic+lat+cro+rec+dut*	0.139	0.118	0.125	0.117	0.090	0.095	0.063	0.077	0.095				
G11	*Borrelia turicatae + parkeri*	0.181	0.178	0.182	0.191	0.145	0.119	0.166	0.141	0.143	0.144			
G12	*Borrelia turcica*	0.247	0.210	0.234	0.212	0.217	0.212	0.193	0.193	0.207	0.178	0.230		
G13	*Borrelia burgdorferi* s.l.	0.226	0.202	0.208	0.205	0.160	0.169	0.160	0.145	0.181	0.155	0.194	0.195	

***glpQ* genetic distances analysis**

		G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14
G1	*Borrelia* sp. Portugal ○														
G2	*Borrelia theileri*	0.090													
G3	*Borrelia* sp. Portugal ● + *Borrelia* sp. Japan	0.065	0.084												
G4	*Borrelia lonestari*	0.093	0.095	0.072											
G5	*Borrelia miyamotoi*	0.118	0.110	0.111	0.088										
G6	*Borrelia turcica*	0.199	0.180	0.176	0.176	0.139									
G7	*Borrelia* sp.	0.180	0.169	0.178	0.174	0.146	0.093								
G8	*Borrelia turicatae*	0.178	0.175	0.184	0.170	0.135	0.097	0.017							
G9	*Borrelia parkeri*	0.223	0.199	0.203	0.186	0.171	0.156	0.176	0.172						
G10	*Borrelia hermsii*	0.222	0.201	0.200	0.184	0.178	0.164	0.180	0.175	0.027					
G11	*Borrelia persica*	0.198	0.191	0.188	0.170	0.157	0.148	0.165	0.157	0.044	0.046				
G12	*Borrelia hispanica*	0.213	0.213	0.205	0.200	0.181	0.150	0.159	0.153	0.113	0.116	0.099			
G13	*Borrelia crocidurae*	0.182	0.205	0.181	0.224	0.220	0.221	0.182	0.201	0.205	0.209	0.215	0.221		
G14	*Borrelia dut+rec+mic*	0.188	0.213	0.187	0.223	0.208	0.191	0.166	0.177	0.213	0.220	0.215	0.222	0.056	

Borrelia hisp+mic+lat+cro+rec+dut – *Borrelia hispanica. microti. latyschewii. crocidurae. recurrentis. duttonii; Borrelia dut+rec+mic* – *Borrelia duttonii. recurrentis. microti;*
Open and closed circles represent, respectively, *Haemaphysalis punctata* and *Rhipicephalus sanguineus* ticks.

Fig. 4. *glpQ* and *flaB* genetic distance analysis calculated using the Tamura–Nei as implemented in the Mega 6.0 software.

still important agents of animal disease, and therefore should be characterized more thoroughly.

The results presented in the current study confirm previous reports indicating a countrywide distribution of *B. burgdorferi* s.l. bacteria in questing ticks (De Michelis et al., 2000; Baptista et al., 2004). In one of these studies *B. lusitaniae* was the most prevalent species in the questing ticks (Baptista et al., 2004). Nevertheless, other *B. burgdorferi* s.l. species were also found throughout Portugal, even in ticks other than their "classical" known *I. ricinus* vector (De Michelis et al., 2000; Baptista et al., 2004; Núncio and Alves, 2014). The presence of spirochetes in these ticks does not necessarily mean that they are capable of transmitting them to new hosts. Tick species without the ability of transmission of the pathogen should not be recognized as vector competent. They can be called non-vector species. Examples of these non-vector are *Dermacentor reticulatus*, *D. andersoni*, *D. variabilis*, *D. occidentalis*, *Haemaphysalis concinna* and *H. punctata*. None of these ticks are recognized as vectors of *Borrelia* for their inability of transstadial transmission in natural conditions (Gern et al., 1991; Kahl et al., 1992; Barbour and Fish, 1993; Angelov et al., 1996; Dolan et al., 2000; Sun and Xu, 2003).

Somewhat unexpectedly, this study, backed by phylogenetic analysis of the DNA sequences of three independently amplified genetic markers (16S rRNA, *flaB* and *glpQ*), also suggested the existence of two possible new RF spirochetes, presently referred to as *Borrelia sp.*, detected in *H. punctata* and *R. sanguineus* questing ticks.

H. punctata ticks comprised five pools of larvae collected in July 2012, as well as one nymph captured later in the same year (December) at Tapada Nacional de Mafra, near Lisboa. This is a protected area managed mainly for the sustainable use of natural ecosystems, composed by dense forests of deciduous oaks, pines, eucalyptus, chestnuts and *Platanus* trees, and inhabited by numerous mammal species. These include fallow deer (*Cervus dama*), red deer (*Cervus elaphus*), wild boar (*Sus scrofa*), foxes (*Vulpes vulpes*), wild-rabbits (*Oryctolagus cuniculus*), European hedgehogs (*Erinaceus europaeus*), as well as many bats and small rodents. This wide range of hosts allows the maintenance of permanent populations of numerous species of ticks, thus contributing to the persistence of several species of *Borrelia* in this protected biotope.

The data presented here report the detection of RFB-like *Borrelia* in ticks at two different development stages, most of which being larvae. Although this is compatible with the idea that these bacteria might be transmitted transovarially, how the larvae became infected in the first place remains undetermined. Spirochetes may have either originated from an interrupted blood meal (with the ticks failing further development) or may have already been present in the bodies of immature specimens at the moment of egg hatching due to transovarial transmission (TOT). The latter hypothesis has been explored for many tick-borne pathogens for maintenance in natural environment and can occur in both ixodid and argasid ticks (Rollend et al., 2013). The presence of RFB such as *B. miyamotoi* in larvae has not only been shown to result from direct transovarial acquisition from infected female ticks, but these larvae have also been proven competent for transmission of these spirochetes to vertebrates (Lee et al., 2014).

Two adult females of *R. sanguineus* have also been shown to harbor RFB-DNA. One of these ticks was captured in July of 2012 in the north of Portugal Braga district) in a wetland region surrounded by mountains with forests inhabited by several large mammal species like red deer, fallow deer, and wild boar. The other tick was captured in April of 2014 in the south of Lisboa (near Évora), in a biotope characterized by drier environmental conditions, with arid lands, high temperatures in the summer, and where roaming bovines are frequently observed. The fact that two ticks harboring RFB-DNA were caught at collection sites located hundreds of kilometers apart, suggest a possible wider distribution of these bacteria.

Interestingly, phylogenetic analyses of 16S rRNA, *flaB* and *glpQ* sequences have revealed generally congruent tree topologies in which the novel RFB sequences detected in this study form two independent clusters, consistently supported by bootstrap and posterior probability values. These clusters placed the analyzed sequences in a larger subgroup of RFB that included *B. theileri*, *B. lonestari*, and a number of unclassified spirochetes, referred to as *Borrelia* sp. Furthermore, they were clearly independent from one another, possibly revealing two different species of RFB. DNA of each of the two new RFB species was detected in a single tick species, either *H. punctata* or *R. sanguineus*. The *Borrelia* sequences amplified from *H. puntacta* was only detected in specimens collected at Tapada Nacional de Mafra. However, whether this apparent restricted distribution results from sampling bias, or suggests maintenance of these bacteria solely within the confinements of the Tapada Nacional de Mafra habitat, remains to be determined. Potentially, whether transmission restricted to certain tick species is also an open question for concerning *Borrelia sp.* detected in *R. sanguineus*. Due to the small number of positive detections of this type of RFB, it is not clear if these bacteria are restricted, or not, to the species of ticks where they have been found. However, phylogenetic analysis of *flaB* and *glpQ* sequences suggest a common ancestry between these bacteria and *Borrelia* sp. detected in *Haemaphysalis* spp. from Japan (Lee et al., 2014), which seems to contradict the idea of tick-restriction, but clearly deserves to be explored in the future. The observed genetic heterogeneity within the G1–G4 cluster (Supplementary Fig. 1), revealed by both phylogenetic and genetic distance analyses (Fig. 4), suggests that it may be formed by as many as four different *Borrelia* species (based on currently available data). Clearly, a more thorough genetic characterization of this cluster is worth being done in the near future, possibly involving new approaches such as multilocus sequence typing. These will be of importance to aid in the definition of clear-cut genetic limits for assignment of monophyletic groups of *Borrelia* sequences as individual species. Finally, despite the possibilities opened by the use of molecular approaches based on PCR, followed by phylogenetic analyses of the obtained DNA sequences, the characterization of these new RFB clearly calls for the isolation and in vitro cultivation of these bacteria. No attempts were made to isolate *Borrelia* from the ticks collected during this work as they were conserved in ethanol until further identification, and bacterial isolation was not one of the aims of this study when it was devised. Nevertheless, and despite the anticipated difficulties, the isolation of these bacteria in vitro, their characterization (both genetic and phenotypic) as well as their role in human or veterinary disease, will be the focus of future research.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2016.03.008.

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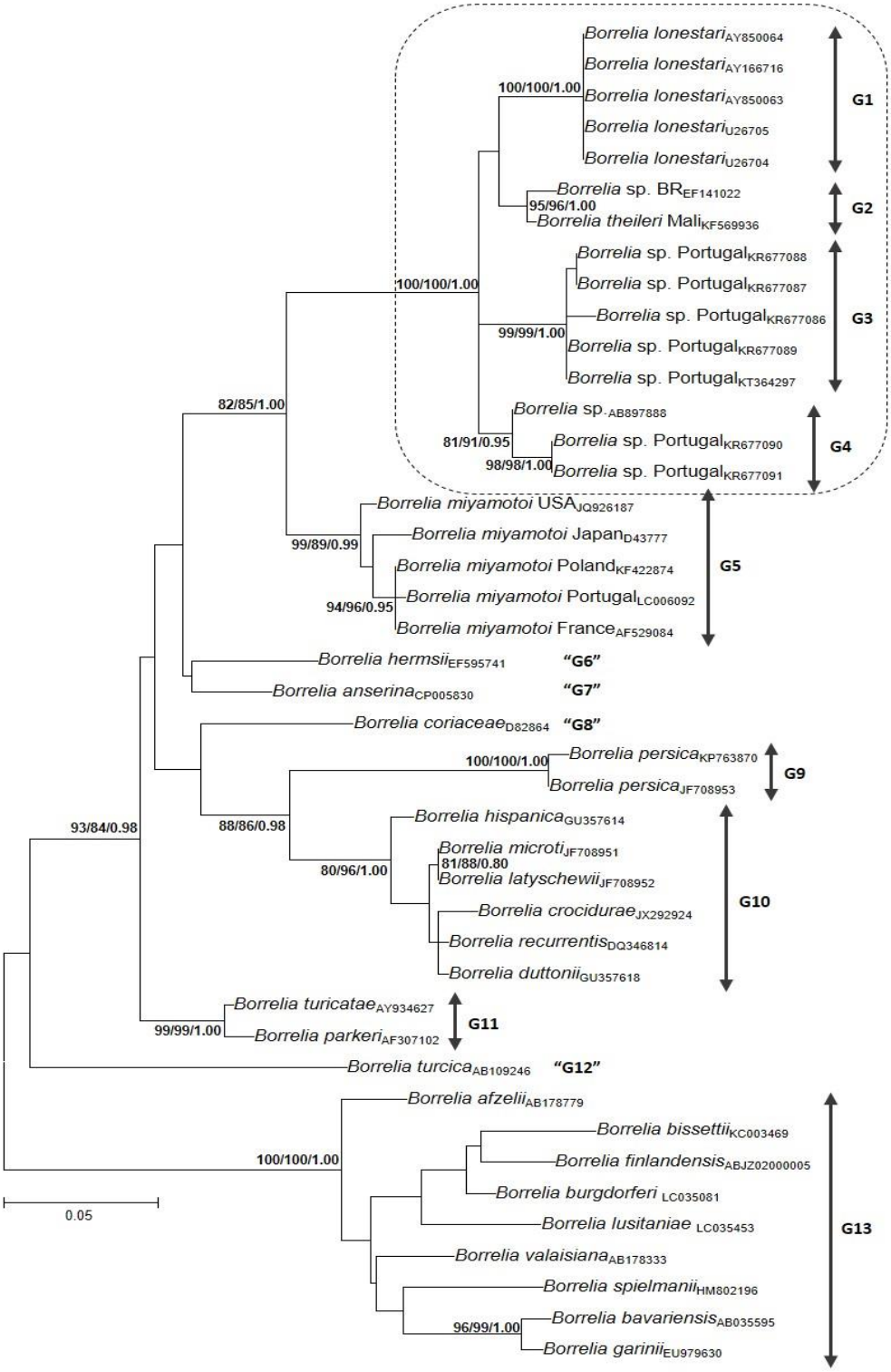
Supplementary data

A



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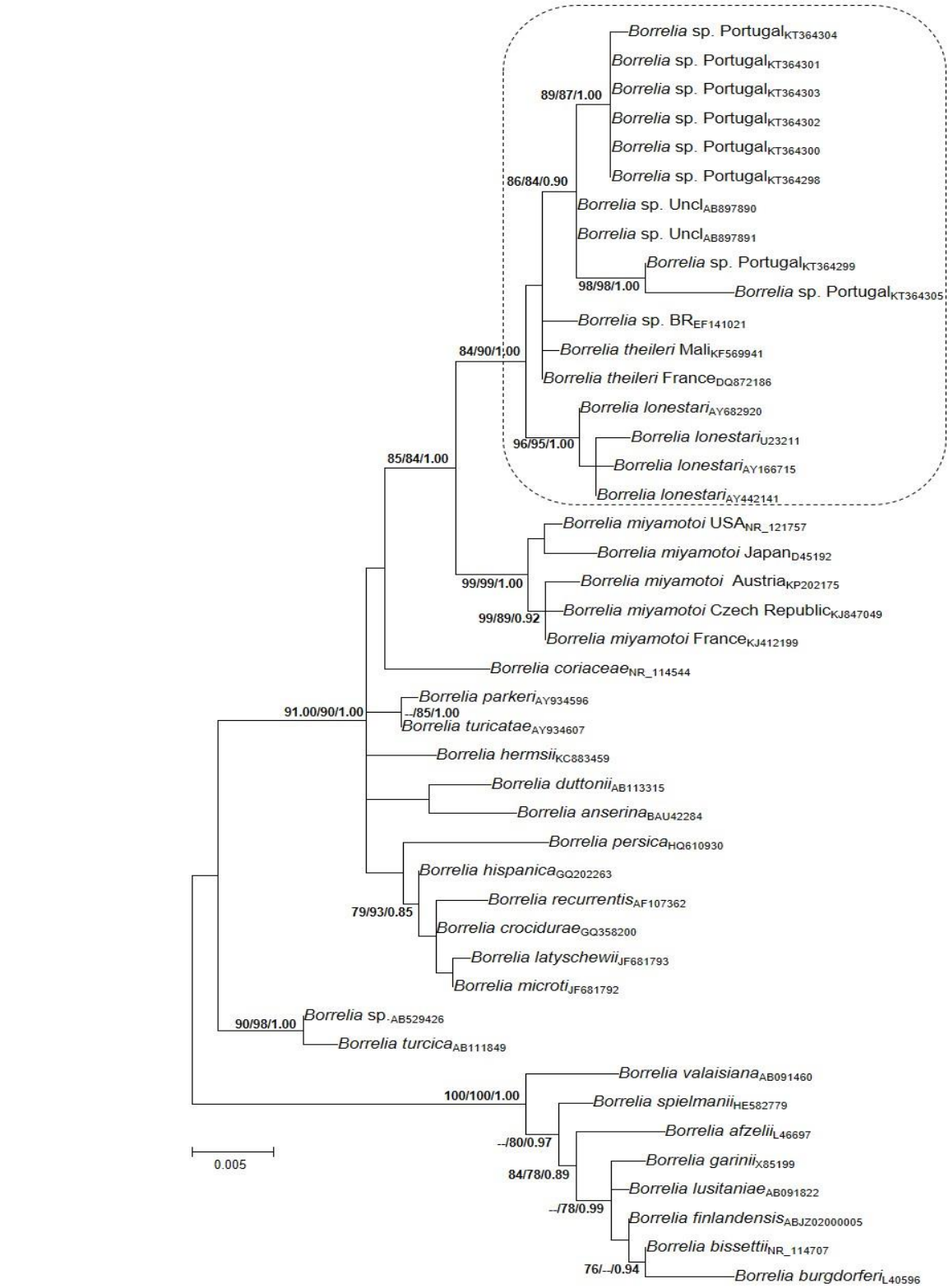
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Borrelia valaisiana AB091460
Borrelia spielmanii HE582779
Borrelia afzelii L46697
Borrelia garinii X85199
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Borrelia finlandensis ABJZ02000005
Borrelia bissettii NR_114707
Borrelia burgdorferi L40596
89/87/1.00
86/84/0.90
98/98/1.00
84/90/1.00
96/95/1.00
85/84/1.00
99/99/1.00
99/89/0.92
91.00/90/1.00
--/85/1.00
79/93/0.85
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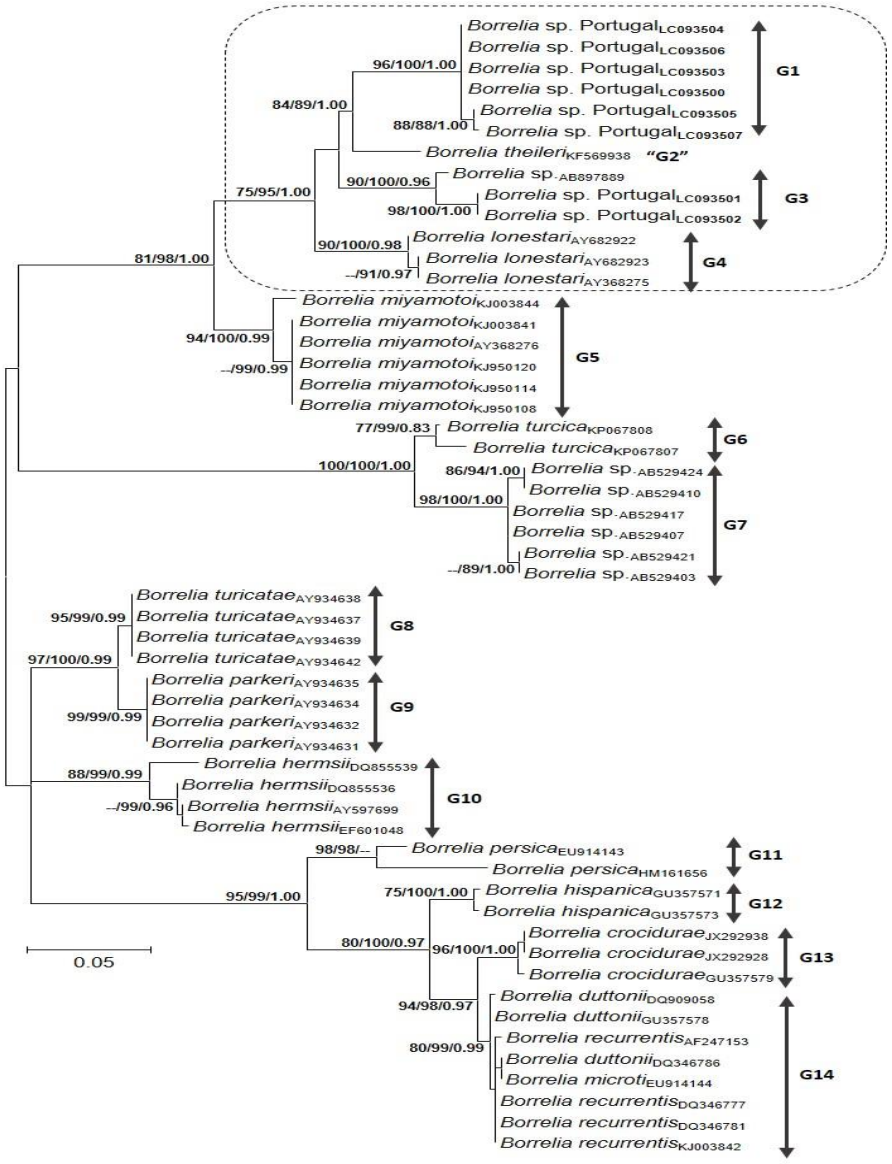
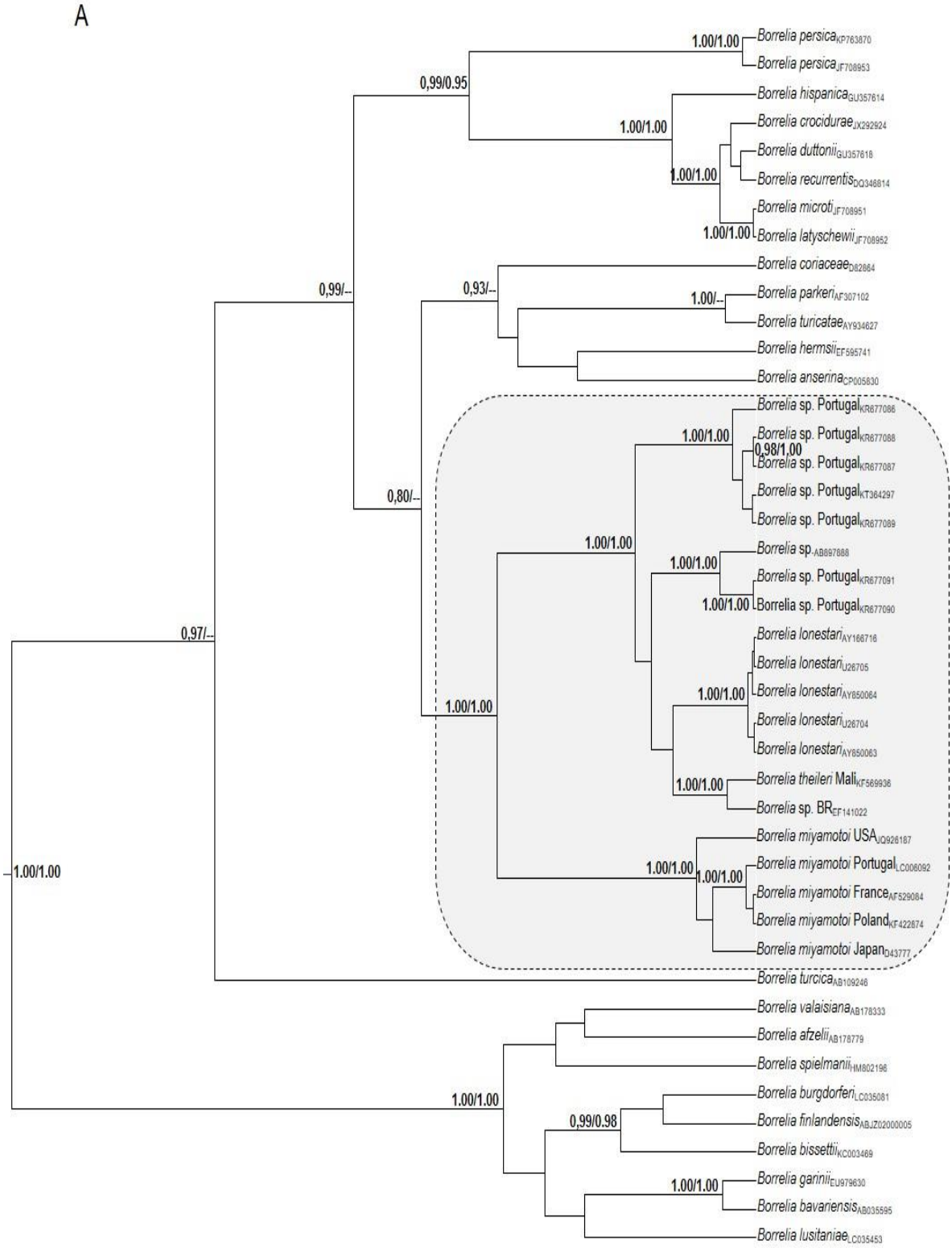


Supplementary Fig. 1.

Complete phylogenetic analysis of Relapsing Fever *Borrelia flaB* (A), 16S rRNA (B), and *glpQ* (C) sequences (partial trees are shown in Fig. 3). At specific branch nodes bootstrap values (b) ≥ 75% or posterior probabilities (pp) ≥ 0.92 are indicated (bNJ/bML/ppBayes). Bootstrap/posterior probability values below these limits are indicated by "--". The size bar indicates the number of nucleotide substitutions per site. Open and closed circles represent, respectively, sequences amplified from*Haemaphysalis* spp. and *Rhipicephalus* spp. ticks. Triangles indicate sequences amplified from sika deer.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Borrelia miyamotoi USA JQ926187
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Borrelia miyamotoi Poland KF422874
Borrelia miyamotoi Japan D43777
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Borrelia valaisiana AB178333
Borrelia afzelii AB178779
Borrelia spielmanii HM802196
Borrelia burgdorferi LC035081
Borrelia finlandensis ABJZ02000005
Borrelia bissettii KC003469
Borrelia garinii EU979630
Borrelia bavariensis AB035595
Borrelia lusitaniae LC035453
1.00/1.00
0,99/0.95
1.00/1.00
1.00/1.00
1.00/1.00
0,99/--
0,93/--
1.00/--
0,80/--
1.00/1.00
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1.00/1.00
1.00/1.00
1.00/1.00
1.00/1.00
0,97/--
1.00/1.00
1.00/1.00
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1.00/1.00
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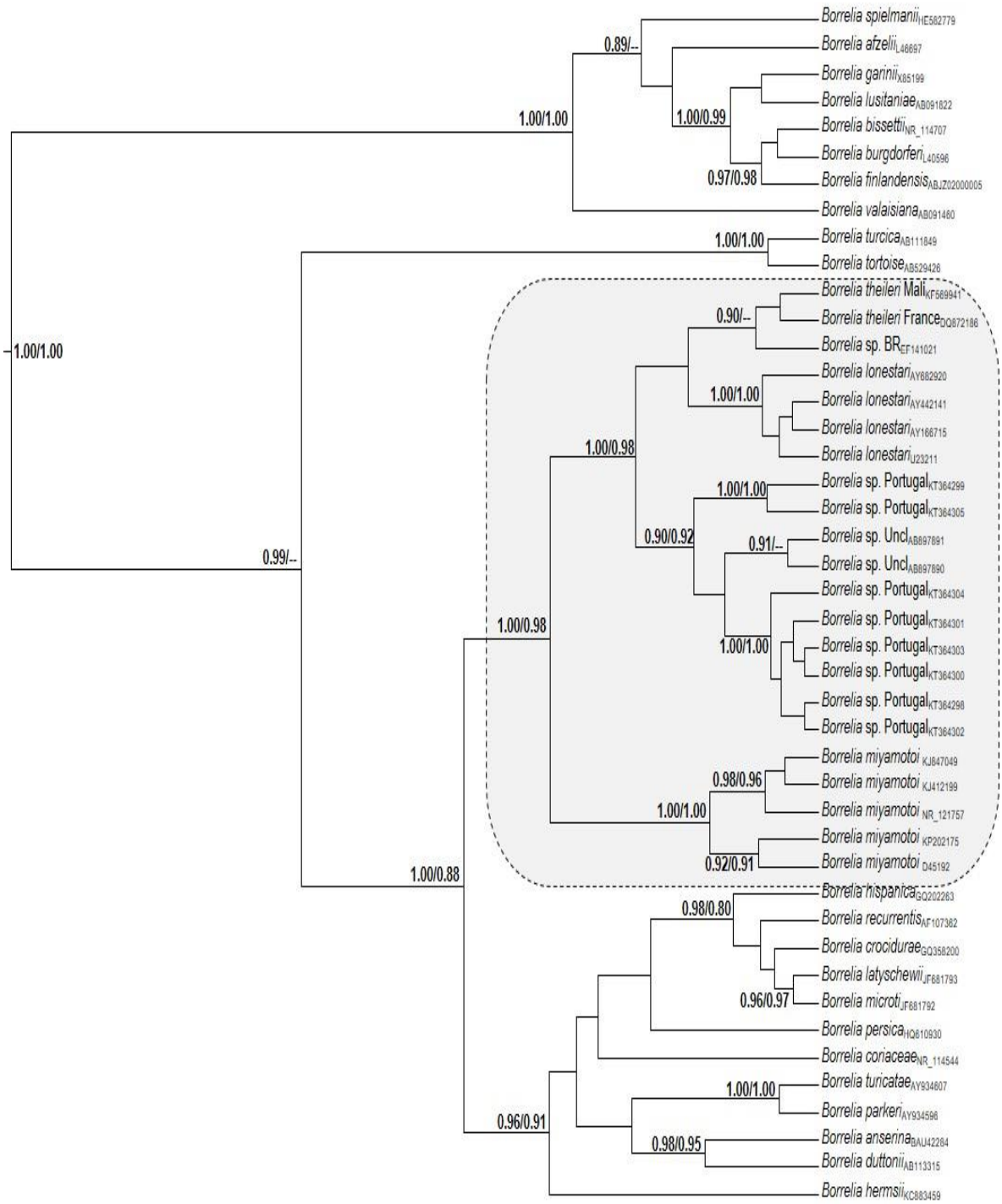
0,99/0.98
1.00/1.00

B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Borrelia valaisiana AB091480
Borrelia turcica AB111849
Borrelia tortoise AB529426
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Borrelia theileri France DQ872186
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Borrelia hermsii KC883459
0.89/--
1.00/1.00
1.00/0.99
0.97/0.98
1.00/1.00
0.90/--
1.00/1.00
1.00/0.98
1.00/1.00
0.90/0.92
0.91/--
1.00/1.00
0.99/--
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1.00/1.00
0.98/0.96
1.00/1.00
0.92/0.91
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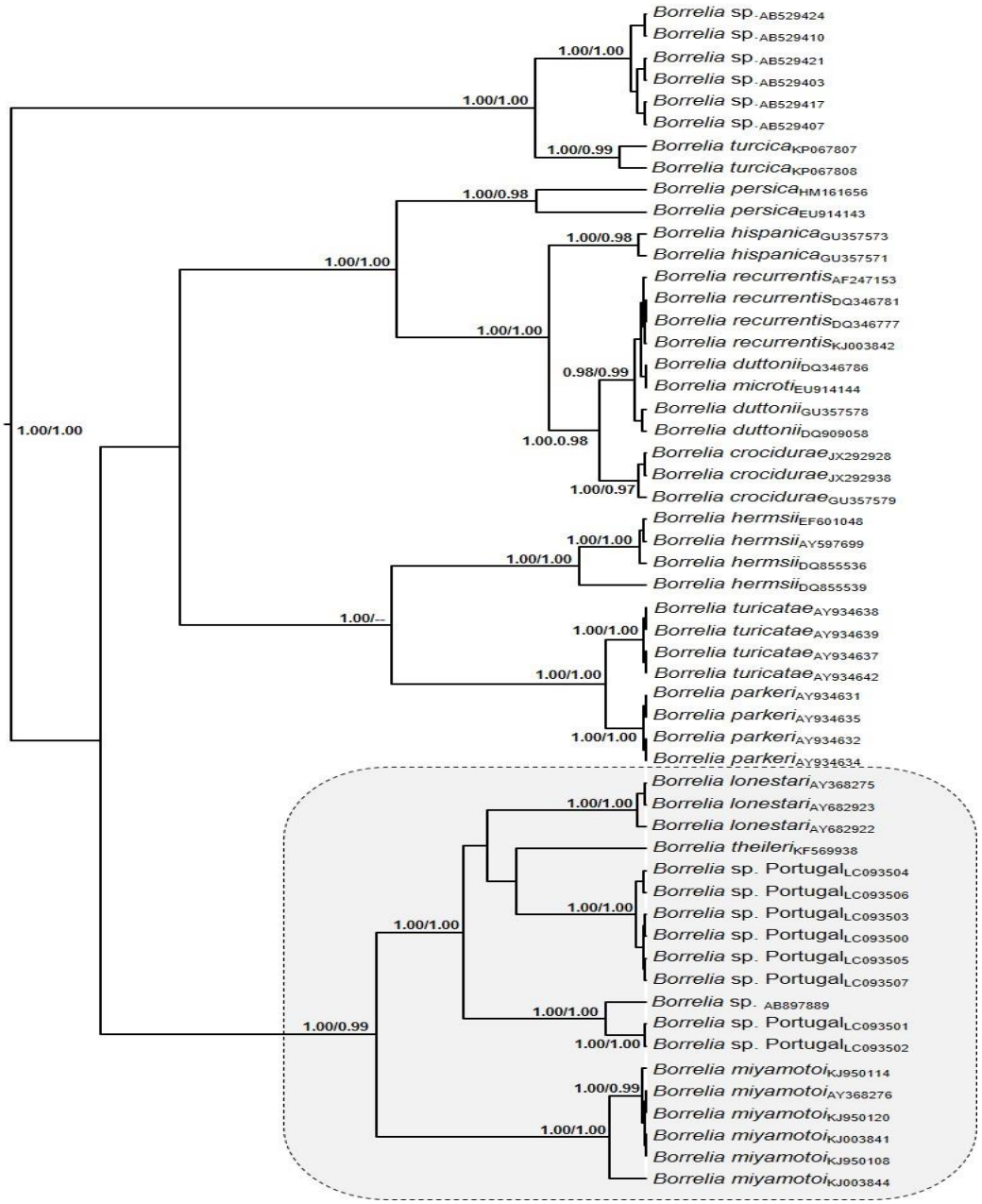
0.96/0.97
1.00/1.00
0.96/0.91
0.98/0.95

C



Supplementary Fig. 2.

Maximum Clade Probability Trees based on the analysis of Relapsing Fever *Borrelia flaB* (A), 16S rRNA (B), and glpQ (C) sequences. At specific branch nodes posterior probabilities (pp) ≥ 0.80 are indicated. The pair of pp values (pp1/pp2) indicates those obtained on the bases of assuming, as coalescent priors, a strict (pp1) or uncorrelated lognormal relaxed clock (pp2). Posterior probability values below these limits are indicated by "--". Sequences boxed in gray encompass those referred to in the text as Relapsing Fever *Borrelia*.

16S rRNA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Borrelia sp. Portugal KT364301
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Borrelia sp. Uncl AB897890
Borrelia sp. Uncl AB897891
Borrelia sp. Portugal KT364299
98
Borrelia sp. Portugal KT364305
Borrelia sp. BR EF141021
83
Borrelia theileri Mali KF569941
Borrelia theileri France DQ872186
Borrelia lonestari AY682920
Borrelia lonestari AY442141
97
Borrelia lonestari AY166715
77
Borrelia lonestari U23211
Borrelia miyamotoi USA NR_121757
Borrelia miyamotoi Japan D45192
Borrelia miyamotoi France KJ412199
99
Borrelia miyamotoi Austria KP202175
82
Borrelia miyamotoi Czech Republic KJ847049
Borrelia coriaceae NR_114544
91
Borrelia parkeri AY934596
Borrelia turicatae AY934607
Borrelia hermsii KC883459
Borrelia duttonii AB113315
Borrelia anserina BAU42284
Borrelia persica HQ610930
Borrelia hispanica GQ202263
Borrelia crocidurae GQ358200
94
Borrelia recurrentis AF107362
Borrelia microti JF681792
Borrelia latyschewii JF681793
Borrelia sp. AB529426
99
Borrelia turcica AB111849
Borrelia valaisiana AB091460
Borrelia spielmanii HE582779
100
Borrelia afzelii L46697
89
Borrelia garinii X85199
79
Borrelia lusitaniae AB091822
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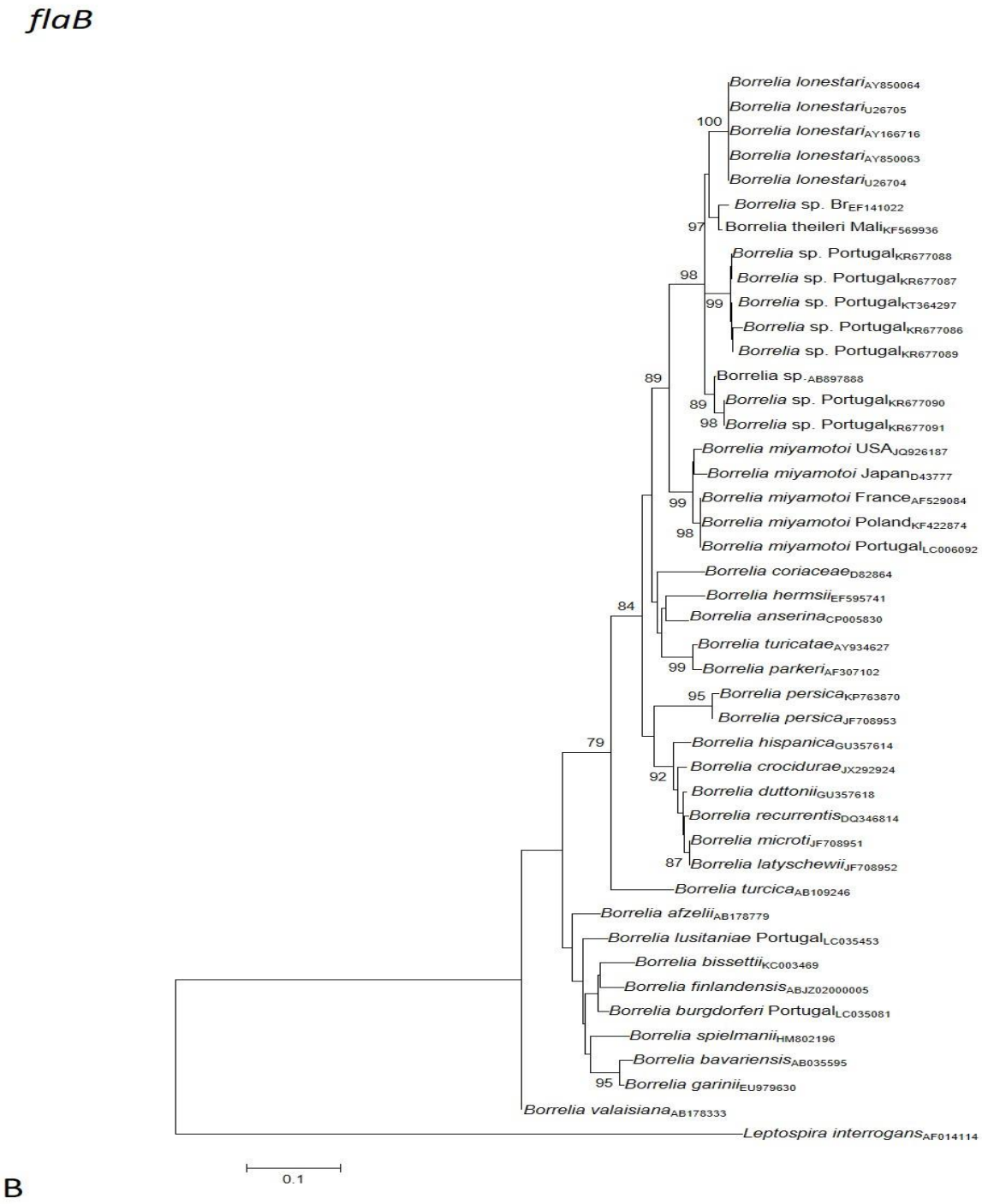
Leptospira interrogans X17547
0.02

A



Supplementary Fig. 3.

Complete phylogenetic analysis of Relapsing Fever *Borrelia* 16S rRNA (A) and *flaB*(B), sequences with the introduction of *Leptospira interrogans* as an outgroup. At specific branch nodes bootstrap values (b) ≥ 75% are indicated (bNJ).

3.2 - Molecular identification of *Borrelia miyamotoi* in *Ixodes ricinus* from Portugal

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Molecular Identification of *Borrelia miyamotoi* in *Ixodes ricinus* from Portugal

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Abstract

Borrelia miyamotoi, a relapsing fever spirochete, has been found recently in *Ixodes ricinus* ticks; however, little is known about its spatial distribution and potential local impact on human health. A total of 640 ticks (447 nymphs and 193 adults) collected throughout Portugal were analyzed using two nested PCR protocols, one targeting the flagellin gene and the other the internal transcribed space region between the 5S and the 23S rRNA. As a result, *B. miyamotoi* was detected, for the first time, in one guesting *I. ricinus* nymph collected in the Lisboa district. In addition, a prevalence of 11% (71/640) for *B. burgdorferi* sensu lato was obtained. Even though no human relapsing fever cases due to infection by *B. miyamotoi* have been reported yet in Portugal, surveillance must be improved to provide better insight into the prevalence and distribution of this spirochete in ticks.

Key Words: *Borrelia miyamotoi*—*Ixodes ricinus*—Flagellin gene—Portugal.

Introduction

IXODES RICINUS IS THE MOST widespread and abundant European tick species capable of transmitting pathogens of medical and veterinary importance. These include bacteria such as *Borrelia burgdorferi* sensu lato (s.l.), considered a prevalent pathogen in these ticks, and causing Lyme borreliosis, *Anaplasma phagocytophilum*, the etiologic agent of human granulocytic anaplasmosis, *Francisella tularensis* causing tularaemia, *Rickettsia helvetica* and *R. monacensis* causing spotted fever rickettsiosis; protozoa such as *Babesia divergens* and *B. microti*, causal agents of babesiosis, and *Candidatus* Neoehrlichia mikurensis, responsible for neoehrlichiosis. This tick species has also been associated with the transmission of a multitude of viruses, including tick-borne encephalitis, Louping ill, and Tribec viruses (Medlock et al. 2013).

Other *Borrelia* spp., such as *Borrelia miyamotoi*, belonging to the relapsing fever group have recently been detected in *I. ricinus* ticks. This species was first identified in 1995 by Fukunaga and collaborators in *Ixodes persulcatus* ticks and in a small mammal (*Apodemus argenteus*), at Hokkaido, Japan (Fukunaga et al. 1995). Since then, *B. miyamotoi* has been found in several ticks classified in the *Ixodes* genus in North America, Europe, and Asia, revealing an extensive geographic distribution (Geller et al. 2012, Cochez et al. 2014, Cosson et al. 2014, Dibernardo et al. 2014, Hansford et al., 2014, Kiewra et al. 2014, Takano et al. 2014).

The aim of the present study was to determine the presence of *B. miyamotoi* in questing *I. ricinus* ticks collected throughout Portugal and to evaluate the phylogenetic relatedness of these spirochetes to other species of the relapsing fever group.

Material and Methods

To assess the presence of *B. miyamotoi* in *I. ricinus* ticks from Portugal, 12 sites from six districts from the North (Braga and Vila Real), Center (Aveiro, Lisboa and Setúbal) and South (Faro) regions of Portugal were selected (www.mapas-portugal.com/Mapa_Distritos_Portugal.htm). Between May, 2012, and May, 2014, from spring to autumn, ticks were collected by flagging once a month, conserved in 70% ethanol, and identified at the species level using taxonomic keys (Estrada-Peña et al. 2004). For logistic reasons, given the proximity to our laboratory, the majority of ticks were collected in the Lisboa district.

Ticks' genomic DNA was extracted by adding 500 μL of ammonium hydroxide (NH_4OH) to each adult tick, and 100 μL to each immature stage (nymphs). Adult specimens and

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TABLE 1. DISTRICT, STAGE, AND NUMBER OF *IXODES RICINUS* TICKS COLLECTED IN PORTUGAL AND ANALYZED FOR THE PRESENCE OF *BORRELIA MIYAMOTOI* AND *B. BURGDORFERI* SENSU LATO[a]

	Ticks examined			*Infected ticks by Borrelia spp.*[b]	
District	*Stage*	*No.*	*No. infected*	*B. burgdorferi s.l. (%)*	*B. miyamotoi (%)*
Braga	Adult	2			
Vila Real	Adult	16	3	2	
Aveiro	Adult	3			
Lisboa	Nymph	447	50	24	1
	Adult	118	2	0	
Setúbal	Adult	19	3		
Faro	Adult	35	13	7	
Total		640	72	33	1

[a]Ticks were collected from several different locations within the six surveyed districts.
[b]Sequencing results from 47% of the positive tick samples.

nymphs were processed individually. The lysates obtained were then stored at −20°C for further use.

Detection of *Borrelia* DNA was carried out using two different nested PCR protocols. The first protocol targeted the internal transcribed spacer (ITS) region between the 5S and 23S rDNA with the external primers 23SN1 and 23SC1 (amplifying a 320-bp fragment) and inner primers 23SN2 and 5SC (amplifying a 280-bp fragment). The PCR conditions were denaturation at 94.5°C for 1 min, 25 cycles of amplification at 94°C for 30 s, 52°C for 30 s (outer primers) or 55°C for 30 s (inner primers), and 72°C for 1 min, followed by a 5-min extension phase at 72°C.

The second nested PCR targeted the flagellin gene and included a first amplification with the outer primers 132f and 905r (amplifying a 774-bp fragment) and a second amplification using the inner primers 220f and 823r (amplifying a 604-bp fragment). The PCR conditions were denaturation at 94°C for 10 min, 40 cycles of amplification at 94°C for 30 s, 50°C for 45 s (outer primers) or 54°C for 45 s (inner primers), and 72°C for 1 min, followed by a 7-min extension phase at 72°C.

The PCR products were directly sequenced at GATC Biotech AG (Cologne, Germany), and the sequences were compared with those available in the GeneBank/EMBL/DDBJ database using the National Center for Biotechnology Information (NCBI) BLAST program. Additionally, phylogenetic trees were constructed using the neighbor-joining (NJ) clustering algorithm and a matrix of corrected genetic distances or inferred using the maximum likelihood (ML) criterion, starting from a NJ tree, using Mega 5.0 (www.megasoftware.net/). Correction of distances (for NJ analysis) and phylogenetic tree search based on the ML optimization criterion were carried out using the Tamura–Nei + Γ model, as suggested by Mega 5.0. In both cases, the topology of the trees obtained was assessed by bootstrapping, with 1000 random resamplings or the original sequence data. Phylogenetic analysis also included the construction of a tree following a Bayesian approach using the MrBayes v3.0b4 software (http://mrbayes.sourceforge.net/).

Results

A total of 640 *I. ricinus* ticks were collected and analyzed according to the following distribution by district: Braga, two adults; Vila Real, 16 adults; Aveiro, three adults; Lisboa, 447 nymphs and 118 adults; Setúbal, 19 adults; and Faro, 35 adults (Table 1).

Analysis of nucleotide sequences of the *fla* gene amplicons obtained from a nymph collected in the district of Lisboa (Tapada Nacional de Mafra) and deposited in the GenBank/EMBL/DDBJ databases under accession number LC006092 revealed 99% identity with several *B. miyamotoi* reference sequences (BLASTn). Phylogenetic trees constructed on the basis of the analysis of nucleotide sequence alignments of *fla* coding sequences (using multiple *Borrelia* spp. as references) clearly indicate that LC006092 (indicated by the arrow in Fig. 1), clustered with the group of relapsing fever–like *Borrelia* within the European sequence lineage (supported by bootstrap analysis of the NJ and ML trees). This lineage, found inside the *B. miyamotoi* monophyletic group, segregates from the Japan/Russia–Asia, and US monophyletic clusters.

Finally, *B. burgdorferi* s.l. DNA was also amplified by the two nested PCR protocols, leading to an overall prevalence of 11% (71/640). These positive samples were collected from Vila Real, Lisboa, Setúbal, and Faro districts, and about 50% (33/71) of them were sequenced, *B. lusitaniae* being the predominant genospecies (18/33).

Discussion

In the current study, *B. miyamotoi* DNA was detected for the first time in Portugal in a questing nymph collected in Tapada Nacional de Mafra, in the outskirts of Lisboa. However, so far, no evidence has ever been found to indicate that this putative pathogen may be responsible for causing human clinical cases in the country. Nevertheless, studies from Europe have suggested that *B. miyamotoi* may cause human disease, because *B. miyamotoi*–specific antibodies and DNA have already been detected in patients presenting symptoms like fever, headache, and muscle aches, typical of tick-borne relapsing fever.

Current studies have shown a crucial role of several vector species in the spread of *B. miyamotoi*. The typical vectors of tick-borne relapsing fever spirochetes are soft ticks from the genus *Ornithodoros*, therefore the low prevalence of *B. miyamotoi*, revealed by this and other studies may indicate a recent emergence and spread of these bacteria in hard ticks. It is important to highlight that ticks could simultaneously transmit *B. miyamotoi* and Lyme disease spirochetes to humans, supporting the risk of possible co-infections to occur, with potential strong implications for public health. Therefore, further

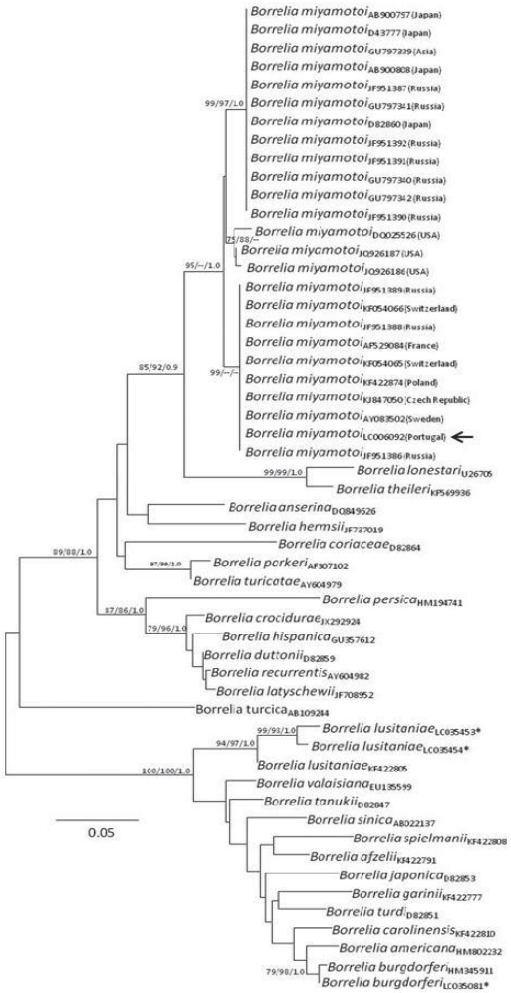

FIG. 1. *Borrelia* phylogenetic analysis of *fla* nucleotide sequences (372 bp). *B. miyamotoi* clusters are denoted by species, accession number, and country of origin. At specific branch nodes bootstrap values (b; >75%) or posterior probabilities (pp; >0.90) are indicated ($b_{NJ}/b_{ML}/pp_{Bayes}$), "–" indicating either bootstrap values or posterior probabilities below the indicated limits of significance. The arrow indicates the position of the *B. miyamotoi*, and the asterisks indicate the positions of other *B.b.*s.l. species, all described in this article. The size bar indicates 5% divergence.

investigations are needed to understand the competence of *I. ricinus* for *B. miyamotoi* and predict a possible spread of this group of spirochetes in Portugal. This will, in turn, contribute to the determination of the risk of exposure (for humans) in areas where *Ixodes* ticks live.

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Author Disclosure Statement

No competing financial interests exist.

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Original article

Molecular detection of bacterial and parasitic pathogens in hard ticks from Portugal

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ABSTRACT

Ticks are important vector arthropods of human and animal pathogens. As information about agents of disease circulating in vectors in Portugal is limited, the aim of the present study was to detect bacteria and parasites with veterinary and zoonotic importance in ticks collected from dogs, cats, and field vegetation. A total of 925 ticks, comprising 888 (96.0%) adults, 8 (0.9%) nymphs, and 29 (3.1%) larvae, were collected in 4 geographic areas (districts) of Portugal. Among those, 620 (67.0%) were removed from naturally infested dogs, 42 (4.5%) from cats, and 263 (28.4%) were questing ticks obtained from field vegetation. *Rhipicephalus sanguineus* was the predominant tick species, and the only one collected from dogs and vegetation, while all *Ixodes ricinus* specimens ($n=6$) were recovered from cats. *Rickettsia massiliae* and *Rickettsia conorii* were identified in 35 ticks collected from cats and dogs and in 3 ticks collected from dogs. Among ticks collected from cats or dogs, 4 *Rh. sanguineus* specimens were detected with *Hepatozoon felis*, 3 with *Anaplasma platys*, 2 with *Hepatozoon canis*, one with *Anaplasma phagocytophilum*, one with *Babesia vogeli*, one with *Borrelia burgdorferi* sensu lato and one with *Cercopithifilaria* spp. *Rickettsia helvetica* was detected in one *I. ricinus* tick collected from a cat. To the best of our knowledge, this was the first time that *Cercopithifilaria* spp., *Ba. vogeli*, *H. canis*, and *H. felis* have been detected in ticks from Portugal. The wide range of tick-borne pathogens identified, some of zoonotic concern, suggests a risk for the emergence of tick-borne diseases in domestic animals and humans in Portugal. Further studies on these and other tick-borne agents should be performed to better understand their epidemiological and clinical importance, and to support the implementation of effective control measures.

Introduction

Ticks are important vector arthropods of human and animal pathogens, including viruses, bacteria, and parasites, some of which have zoonotic importance (Dantas-Torres et al., 2012). In the past few years, the abundance, range of distribution, and habitat of many species of ticks and the prevalence of tick-borne pathogens in dogs and cats have increased worldwide (Beugnet and Marié, 2009; Dantas-Torres et al., 2012). In fact, the spread and dissemination of ticks is a public health concern, especially in areas where domestic animals live in close vicinity of their owners, as they might act as reservoir hosts and direct sentinels for human infections (Beugnet and Marié, 2009; Harrus et al., 2011; Dantas-Torres et al., 2012). Molecular assessment of tick-borne pathogens from ticks collected from hosts or field vegetation is a useful method to evaluate the risk of emerging tick-borne diseases in a certain geographic area (Claerebout et al., 2013; Ionita et al., 2013).

The Mediterranean region provides a suitable environment for the development of a wide range of tick species. *Rhipicephalus sanguineus* and *Ixodes ricinus* are distributed throughout Portugal and have been found to feed on a high variety of hosts, including

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humans (Santos-Silva et al., 2011). Mediterranean spotted fever (MSF), caused by Rickettsia conorii and transmitted by *Rh. sanguineus*, is a notifiable disease in Portugal, where it has reached a higher incidence of complicated cases with development of severe vasculitis and multi-organ failure than in other Mediterranean endemic countries such as Italy and Spain (Sousa et al., 2008; Oteo and Portillo, 2012). Rickettsia massiliae, a member of the spotted fever group rickettsiae, has been detected in ticks from several Mediterranean regions, including Portugal, and isolated from humans (Beati and Raoult, 1993; Bacellar et al., 1995; Fernández-de-Mera et al., 2009; Toledo et al., 2009). Other bacteria such as *Anaplasma* spp. and *Borrelia burgdorferi* sensu lato have also been isolated or molecularly/serologically detected in Portuguese patients (Collares-Pereira et al., 2004; Sousa et al., 2008). In addition, several agents of canine and feline tick-borne diseases, such as anaplasmosis, babesiosis, ehrlichiosis, hepatozoonosis, Lyme borreliosis and spotted fever group rickettsioses, have been reported in dogs and cats from Portugal (Alves et al., 2009; Santos et al., 2009a; Alexandre et al., 2011; Cardoso et al., 2012; Vilhena et al., 2013). Furthermore, *Bartonella* spp., *Cercopithifilaria* spp., *Coxiella burnetii*, *Francisella tularensis*, and *Leishmania infantum* have also been identified in engorged and/or non-engorged ticks from Italy, Portugal, and Romania (Chomel et al., 2006; Psaroulaki et al., 2006; de Carvalho et al., 2007; Otranto et al., 2011, 2012; Paduraru et al., 2012; Trotta et al., 2012).

As information about pathogens circulating in ticks from Portugal is limited, the aim of the present study was to detect the presence of bacteria and parasites with veterinary and zoonotic importance in ticks collected from dogs, cats, and field vegetation.

Materials and methods

Tick collection and identification

From May 2012 to May 2013, ticks were directly removed from dogs and cats living in farms, in shelters, or attending veterinary medical centres and preserved in 70% alcohol. Collections were made in 4 districts of Portugal, namely Guarda, Lisboa, Setúbal, and Faro (Fig. 1). In June 2012, ticks were also collected by dragging over vegetation during 30 min, for 3 consecutive days, in the district of Faro. Ticks were identified to the species level, with developing stage and gender recorded, using standard morphological keys (Travassos-Dias, 1994).

DNA extraction

After tick species identification, each unfed adult, half of each engorged female (longitudinally divided into 2 halves with sterile forceps and surgical blade), each nymph, and pools of larvae (4–13 larvae per pool) were processed for molecular analysis as previously described (Baptista, 2006). Briefly, the ticks were taken from the 70% ethanol solution, air dried, mechanically crushed in a 1.5-ml safe-lock tube with a bended sterile pipette tip and boiled for 20–25 min in 100-μl aliquots (nymphs and larvae) and 500-μl aliquots (adults) of freshly prepared solution of 1:20 ammonium hydroxide, in order to release DNA. After cooling down, the vial with the lysate was left open and boiled until the liquid was reduced to half of its initial volume (50 μl and 250 μl, respectively). For controlling contamination in this step, three 1.5-ml safe-lock tubes were prepared with only 100 μl of ammonia, and the former procedures were also carried out. The tick lysate was stored at −20 °C until use.

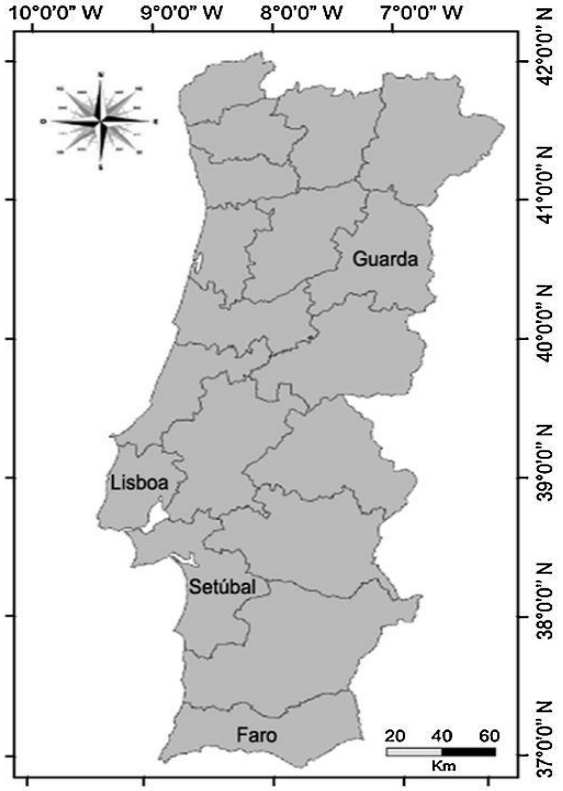

Fig. 1. Map of Portugal depicting the 4 districts from where ticks were collected.

PCR amplification and sequencing

In order to detect false-negative results due to PCR inhibition and to validate the efficiency of the DNA extraction, a PCR targeting the tick 16S rDNA gene was performed (Rich et al., 1995). Optimal amplification conditions were as follows: 34 cycles of denaturation at 93 °C for 30 s, annealing at 45 °C for 60 s, and extension at 72 °C for 3 min. Amplification was completed by an additional cycle at 72 °C for 6 min.

A 350-bp fragment of the tick mitochondrial 16S rRNA gene was amplified in all DNA tick samples.

The presence of *Anaplasma* spp./*Ehrlichia* spp., *Babesia* spp., *Bartonella* spp., *Bo. burgdorferi* s.l., *Cercopithifilaria* spp., *Hepatozoon* spp., *L. infantum*, and *Rickettsia* spp. DNA in ticks was tested by PCR, with the primers and PCR conditions described in Table 1, according to previously described protocols (Regnery et al., 1991; Schwartz et al., 1992; Cortes et al., 2004; Diniz et al., 2007; Harrus et al., 2011; Otranto et al., 2011).

PCR amplifications were performed in a 25-μl final volume reaction containing 2 mM $MgCl_2$, 1 unit of Taq DNA polymerase (GoTaq DNA Polymerase®, Promega, USA), 10 pmol of each primer (15 pmol and 50 pmol in the case of *Leishmania* spp. and *Cercopithifilaria* spp., respectively), 0.2 μM (each) dATP, dTTP, dCTP, and dGTP (Dntps set®, Bionline, Citomed, UK), and 3 μl of DNA template (5–200 ng).

Table 1
Primer sets and PCR conditions for DNA amplification and sequencing of pathogens in ticks.

Pathogen	Primers	Product size (bp)	PCR conditions	Reference
Anaplasma spp./*Ehrlichia* spp.	EHR16SD: 5′-GGT ACC YAC AGA AGA AGTCC-3′ EHR16SR: 5′-TAG CAC TCA TCG TTT ACAGC-3′	345	95 °C, 5 min; 35 cycles [94 °C 30 s, 55 °C 30 s, 72 °C 90 s]; 72 °C, 5 min	Harrus et al. (2011)
Babesia spp.	PIRO-A: 5′-AAT ACC CAA TCC TGA CACAGG G-3′ PIRO-B: 5′-TTA AAT ACG AAT GCC CCCAAC-3′	400	94 °C, 3 min; 35 cycles [94 °C 30 s, 64 °C 45 s, 72 °C 30 s]; 72 °C, 7 min	Harrus et al. (2011)
Bartonella spp.	325s: 5′-CTTCAGATGATGATCCCAAGCCTTCTGGCG-3′ 1100as: 5′-GAACCGACGACCCCCTGCTTGCAAAGCA-3′	500–800	95 °C, 5 min; 55 cycles [95 °C 15 s, 66 °C 15 s, 72 °C 15 s]; 72 °C, 1 min	Diniz et al. (2007)
Borrelia burgdorferi sensu lato	Outer primers: 23SN1: 5′-ACCATAGACTCTTATTACTTTGAC-3′ 23SC1: 5′-TAAGCTGACTAATACTAATTACCC-3′	380	94.5 °C, 1 min; 25 cycles [94 °C 30 s, 52 °C 30 s, 72 °C 1 min]; 72 °C, 5 min	Schwartz et al. (1992)
	Inner primers: 23SN2: 5′-ACCATAGACTCTTATTACTTTGACCA-3′ 5SCB: 5′-GAGAGTAGGTTATTGCCAGGG-3′	225	94.5 °C, 1 min; 40 cycles [94 °C 30 s, 55 °C 30 s, 72 °C 1 min]; 72 °C, 5 min	
Cercopithifilaria spp.	Cbcox1F: 5′-CGGGTCTTTGTTGTTTTTATTGC-3′ NTR: 5′-ATAAGTACGAGTATCAATATC-3′	304	95 °C, 10 min; 40 cycles [95 °C 30 s, 58 °C 60 s, 72 °C 60 s]; 72 °C, 1 min	Otranto et al. (2011)
Hepatozoon spp.	HEP-F: 5′-ATA CAT GAG CAA AAT CTC AAC-3′ HEP-R: 5′-CTT ATT ATT CCA TGC TGC AG-3′	626–666	95 °C, 5 min; 34 cycles [95 °C 20 s, 55 °C, 30 s, 72 °C, 90 s]; 72 °C, 5 min	Inokuma et al. (2002)
Leishmania infantum	MC1: 5′-GTTAGCCGATGGTGGTCTTG-3′ MC2: 5′-CACCCATTTTTCCGATTTTG-3′	447	94 °C, 2 min; 30 cycles [94 °C 20 s, 60 °C, 20 s, 72 °C, 30 s]; 72 °C, 5 min	Cortes et al. (2004)
Rickettsia spp.	Rsfg877: 5′-GGG GGC CTG CTC ACG GCG G-3′ Rsfg1258: 5′-ATT GCA AAA AGT ACA GTG AAC A-3′	381	94 °C, 3 min; 35 cycles [95 °C 20 s, 48 °C, 30 s, 60 °C, 120 s]; 72 °C, 7 min	Regnery et al. (1991)

In all amplifications, a positive control containing genomic target DNA and a negative control without DNA were included. The reaction mixtures were cycled in a Thermo Electron Corporation® Px2 Termal Cycler (VWR, USA). PCR products were visualized under UV illumination after electrophoresis migration on a 1.5% gel agarose stained with 0.2 mg/ml ethidium bromide using a 100-bp DNA ladder as a marker.

PCR products were purified with a High Pure PCR Product Purification Kit (Roche® Mannheim, Germany) according to manufacturer's instructions and directly sequenced (one direction) at Stabvida® (Caparica, Portugal) using the same primers as those used for DNA amplification. Sequences were compared for similarity to sequences in GenBank, using the BLAST programme hosted by NCBI, National Institutes of Health, USA (http://www.ncbi.nlm.nih.gov).

Results

Tick identification

A total of 925 ticks, comprising 888 adults (308 males and 580 females), 8 nymphs, and 29 larvae, was collected from 4 districts of Portugal (Table 2; Fig. 1). Most of the adult ticks were *Rh. sanguineus* ($n = 882$; 99.3%), and 6 (0.7%) were *I. ricinus*; 620 ticks were removed from 116 naturally infested dogs, 42 from 14 cats, and 263 were questing ticks. Ticks from vegetation were only collected in the district of Faro, while no specimen was removed from cats in Setúbal. The range of collected ticks was 1–35 per dog and 1–18 per cat, with 40.9% of the dogs and 42.9% of the cats having only a single specimen. *Ixodes ricinus* specimens were only found on cats.

Pathogen detection

No ticks collected from field vegetation were detected with any of the assessed pathogens. On the other side, 51 adult ticks collected from 16 dogs and 4 cats were found to be positive (Table 3). Sequencing of PCR products and comparison with GenBank revealed that *Rh. sanguineus* ticks were positive for *Ri. massiliae* ($n = 35$; 4.0%), *Ri. conorii* ($n = 4$; 0.5%), *H. felis* ($n = 4$; 0.5%), *H. canis* ($n = 2$; 0.2%), *A. phagocytophilum* ($n = 1$; 0.1%), *A. platys* ($n = 3$; 0.3%), *Ba. vogeli* ($n = 1$; 0.1%), *Bo. burgdorferi* s.l. ($n = 1$; 0.1%) and *Cercopithifilaria* spp. ($n = 1$; 0.1%). In addition, *Ri. helvetica* DNA was detected in one *I. ricinus* tick collected from a cat (Table 3). With the exception of one *Rh. sanguineus* coinfected with *H. felis* plus *Ri. massiliae*, all the other positive ticks were found infected with a single pathogen species. *Bartonella* spp., *Ehrlichia* spp., or *L. infantum* DNAs were not detected in any of the ticks examined.

Overall, *Ri. massiliae* DNA was detected in the 4 studied geographic regions. *Rickettsia conorii* and *Ri. helvetica* were also found in the district of Lisboa. *Hepatozoon felis* was amplified in ticks from Guarda and Faro, whereas *H. canis* was only detected in Setúbal. *Anaplasma platys, Ba. vogeli*, and *Cercopithifilaria* spp. were only detected in Faro, *A. phagocytophilum* in Guarda and *Bo. burgdorferi* s.l. in Setúbal.

Discussion

The present study reveals a considerable diversity of pathogens circulating in ticks collected on cats and dogs from the 4 studied districts of Portugal. It was assumed that most pet, farm, and even shelter dogs had not travelled outside the districts where they were sampled, but that possibility cannot be fully ruled out

Table 2
Numbers of ticks collected in the districts of Guarda, Lisboa, Setúbal and Faro.

District	*Ixodes ricinus*						*Rhipicephalus sanguineus*						*Rhipicephalus* spp.						Total *n* (%)
	Males *n* (%)			Females *n* (%)			Males *n* (%)			Females *n* (%)			Nymphs *n* (%)			Larvae *n* (%)			
	Cat	Dog	Vegetation	Cat	Dog	Vegetation	Cat	Dog	Vegetation	Cat	Dog	Vegetation	Cat	Dog	Vegetation	Cat	Dog	Vegetation	
Guarda	0	0	na	0	0	na	7	17	na	11	25	na	0	0	na	0	0	na	60 (6.5)
Lisboa	0	0	na	4	0	na	6	29	na	8	33	na	0	1	na	0	0	na	81 (8.8)
Setúbal	0	0	na	0	0	na	0	74	na	0	117	na	0	0	na	0	0	na	191 (20.6)
Faro	1	0	0	1	0	0	0	64	111	4	257	119	0	2	5	0	1	28	593 (64.1)
Total	1 (0.1)	0	0	5 (0.5)	0	0	13 (1.4)	184 (19.9)	111 (12.0)	23 (2.5)	432 (46.7)	119 (12.9)	0	3 (0.3)	5 (0.5)	0	1 (0.1)	28 (3.0)	925 (100)

na, not assessed.

Table 3
Pathogens detected by PCR and DNA sequencing in ticks from Portugal according to geographic region and vertebrate host, with DNA Data Bank of Japan (ddbj) accession numbers.

Pathogen[a]	Region	Vertebrate host (no. of ticks)[b]	ddbj accession no.
A. phagocytophilum	Guarda	Cat (1)	AB872954
A. platys	Setúbal	Dog (1)	AB872953
A. platys	Faro	Dog (2)	AB872951; AB872952
Ba. vogeli	Faro	Dog (1)	AB872955
Bo. burgdorferi sensu lato	Setúbal	Dog (1)	AB872956
Cercopithifilaria spp.	Faro	Dog (1)	AB872950
H. canis	Setúbal	Dog (2)	AB872944; AB872949
H. felis	Guarda	Cat (2)	AB872946; AB872947
H. felis	Faro	Dog (1)	AB872945
Ri. conorii	Lisboa	Dog (2)	AB872795; AB872805
Ri. conorii	Faro	Dog (1)	AB872796
Ri. helvetica	Lisboa	Cat (1)	AB872943
Ri. massiliae	Guarda	Cat (5)	AB872465; AB872786; AB872790; AB872803; AB872807
Ri. massiliae	Guarda	Dog (4)	AB872784; AB872788; AB872797; AB872798
Ri. massiliae	Lisboa	Cat (1)	AB872802
Ri. massiliae	Lisboa	Dog (7)	AB872463; AB872464; AB872791; AB872793; AB872799; AB872801; AB872806
Ri. massiliae	Setúbal	Dog (4)	AB872785; AB872808; AB872939; AB872941
Ri. massiliae	Faro	Dog (13)	AB872462; AB872466; AB872787; AB872789; AB872792; AB872800; AB872804; AB872809; AB872810; AB872811; AB872812; AB872940; AB872942
H. felis + *Ri. massiliae*	Faro	Cat (1)	AB872794; AB872948

[a] ≥94% identity with the closest sequence deposited in GenBank.
[b] All the agents were detected in *Rh. sanguineus* ticks, except *Ri. helvetica* which was detected in *I. ricinus*.

for all of them. *Rhipicephalus sanguineus* was the predominant tick species, being the only one collected from dogs and vegetation, while all *I. ricinus* specimens were recovered from cats. *Anaplasma* spp., *Babesia* spp., *Bo. burgdorferi* s.l., *Cercopithifilaria* spp., *Hepatozoon* spp., and *Rickettsia* spp. were detected among the assayed tick population.

Agents of several tick-borne ehrlichial and rickettsial diseases, such as MSF, spotted fever group rickettsiosis, and granulocytic anaplasmosis, are shared by man and companion animals (Otranto et al., 2009). In the present study, 3 *Ri. conorii*-positive ticks were collected from dogs. Molecular detection of this pathogen in the blood of sick dogs together with its high seroprevalence in healthy canine populations have recently been reported in

Portugal (Alexandre et al., 2011) and in other endemic areas of MSF (Levin et al., 2012; Pennisi et al., 2012; Trotta et al., 2012), suggesting that dogs are frequently exposed to Ri. conorii and, therefore, can act as reservoir and/or sentinel hosts for human infection.

Ri. massiliae DNA was also amplified from 35 *Rh. sanguineus* ticks collected from cats and dogs living in the 4 screened regions. As far as we are aware, this was the first time that *Ri. massiliae* has been detected in ticks from cats. Despite the fact that it seems to be quite common to find this pathogen in ticks removed from dogs (Marquez et al., 2008; Trotta et al., 2012; Claerebout et al., 2013), there is no evidence that it can cause illness or that the dog can play a role in the transmission of this bacterium to humans. Furthermore, the role of *Rh. sanguineus* as a vector of *Ri. massiliae* still needs to be determined. *Rickettsia helvetica* was also amplified in one out of the 6 *I. ricinus* specimens recovered from cats. Albeit previously detected in *Ixodes* spp. collected from domestic animals, the clinical importance of this pathogen is as yet uncertain (Boretti et al., 2009; Claerebout et al., 2013).

I. ricinus is one of the proven vectors of *A. phagocytophilum* and *Bo. burgdorferi* s.l. in Europe (Dantas-Torres et al., 2012). However, the detection of the agents of granulocytic anaplasmosis and Lyme borreliosis in *Rh. sanguineus* in this study is not completely surprising, since both pathogens have previously been detected by PCR in different tick species (Baptista, 2006; Toledo et al., 2009). Nevertheless, the epidemiological significance of these findings should be carefully considered as they may only indicate an exposure of the arthropods to the pathogen (Estrada-Peña et al., 2013). In the present study, no ticks collected from field vegetation (i.e. questing ticks) were detected with DNA from any of the pathogens. In fact, the presence of a certain agent in a tick (especially in blood-engorged specimens) might only mean that it ingested some microorganisms with a blood meal taken from an infected host and does not assure that the tick will transmit that agent (Estrada-Peña et al., 2013).

A. platys presumptively transmitted by *Rh. sanguineus* and responsible for canine infectious cyclic thrombocytopenia, an emerging disease in Europe, was detected in 3 of the ticks collected from dogs. Infection with this pathogen was reported from clinically suspected dogs living in the north (Cardoso et al., 2010a) and south of Portugal (Santos et al., 2009b).

To the best of our knowledge, this is the first study in Portugal screening ticks for the presence of protozoa and nematodes with veterinary medical significance. Canine babesiosis or piroplasmosis is a protozoal tick-borne disease with worldwide distribution (Solano-Gallego and Baneth, 2011). Infections with *Ba. canis* (transmitted by *Dermacentor* spp.) and *Ba. vogeli* (transmitted by *Rh. sanguineus*) have been described in dogs from the north (Cardoso et al., 2008) and in cats from the north and centre of Portugal (Vilhena et al., 2013). On the other side, *H. canis* has been confirmed in a dog from northern Portugal (Cardoso et al., 2010b) and *H. felis* in cats from the north and central parts of the country. In the present study, *Ba. vogeli* was detected in Faro, *H. canis* in Setúbal, and *H. felis* in Guarda and Faro, but further studies are needed to better understand the epidemiological and clinical importance of these findings.

This is also the first detection of *Cercopithifilaria* spp. DNA in one *Rh. sanguineus* tick collected from a dog in Portugal. Recent morphological and molecular studies on dermal microfilariae infesting dogs in Italy, Spain, and Greece revealed that they are infected by at least 3 species of the genus *Cercopithifilaria* with *Rh. sanguineus* as their putative vector (Otranto et al., 2011, 2012). Altogether, these data emphasize the need of further evaluation of these filarioids in dogs from Portugal.

Albeit the detection of *Bartonella* spp. and *Leishmania* spp. in ticks had already been reported, the role of ticks in the transmission of these pathogens has yet to be confirmed (Chomel et al., 2006; Otranto et al., 2009; Trotta et al., 2012). In this study, like in the one performed by Toledo et al. (2009), in central Spain, no *Bartonella* or *Leishmania* DNA was found in ticks, indicating that, at least in the surveyed districts, they do not seem to play a role in the cycle of these pathogens.

In conclusion, the wide spectrum of tick-borne pathogens identified in this study, some of them of zoonotic concern, suggests a risk for the emergence of tick-borne diseases in domestic animals and in human beings. Further studies on these and other tick-borne agents should be performed to better understand their epidemiological and clinical importance and to support the implementation of effective control measures.

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Chapter 4

Distribution and diversity of *Borrelia* spirochetes and other tick-borne agents in the hosts

4. Distribution and diversity of *Borrelia* spirochetes and other tick-borne agents in the hosts

Ticks are blood-sucking and opportunistic parasites that can attach to the skin of a variety of vertebrate hosts, having the ability of transmitting a range of pathogens. Domestic animals like cats and dogs play a central role in the transmission cycles of some agents of vector borne diseases (e.g. anaplasmosis, bartonellosis, borreliosis and leishmaniosis) by acting as reservoirs, amplifying hosts or sentinels. Likewise, wildlife animals, like wild boars and cervids, can also harbor a high density of infected ticks, which pathogens can affect the human population as a result of their zoonotic potential. Therefore, the management of these situations calls for a One Health approach, including the increased awareness for human presence, especially in sylvatic environments and areas associated with animal husbandry among veterinarians, physicians and the general public. This chapter describes four studies that assess the presence of tick-transmitted bacteria and protozoa with veterinary and zoonotic importance in domestic and wildlife animals from the North, Centre and South of the Portugal.

This chapter is based on the research papers:

Pereira A, Parreira R, **Nunes M**, Casadinho A, Vieira ML, Campino L, Maia C. 2016. Molecular detection of tick-borne bacteria and protozoa in cervids and wild boars from Portugal. *Parasites & Vectors*, 9, 251. doi: 10.1186/s13071-016-1535-0;

Faria AS, Paiva-Cardoso MN, **Nunes M**, Carreira T, Vale-Gonçalves HM, Veloso O, Coelho C, Cabral JA, Vieira-Pinto M, Vieira ML. 2015. First Detection of *Borrelia burgdorferi* sensu lato DNA in Serum of the Wild Boar (*Sus scrofa*) in Northern Portugal by Nested-PCR. *EcoHealth*, 12(1): 183-87. doi: 10.1007/s10393-014-0973-4;

Maia C, Almeida B, Coimbra M, Fernandes MC, Cristovão JM, Ramos C, Martins A, Martinho F, Silva P, Neves N, **Nunes M**, Vieira ML, Cardoso L, Campino L. 2015. Bacterial and protozoal agents of canine vector-borne diseases in the blood of domestic and stray dogs from southern Portugal. *Parasites & Vectors*, 23 (8): 138. doi:10.1186/s13071-015-0759-8;

Maia C, Ramos C, Coimbra M, Bastos F, Martins A, Pinto P, **Nunes M**, Vieira ML, Cardoso L, Campino L. 2014. Bacterial and protozoal agents of feline vector-borne diseases in domestic and stray cats from southern Portugal. *Parasites & Vectors,* 7 (115): 2 – 8. doi: 10.1186/1756-3305-7-115.

4.1 – Molecular detection of tick-borne bacteria and protozoa in cervids and wild boars from Portugal

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Parasites & Vectors

SHORT REPORT Open Access

Molecular detection of tick-borne bacteria and protozoa in cervids and wild boars from Portugal

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Abstract

Background: Wildlife can act as reservoir of different tick-borne pathogens, such as bacteria, parasites and viruses. The aim of the present study was to assess the presence of tick-borne bacteria and protozoa with veterinary and zoonotic importance in cervids and wild boars from the Centre and South of Portugal.

Methods: One hundred and forty one blood samples from free-ranging ungulates including 73 red deer (*Cervus elaphus*), 65 wild boars (*Sus scrofa*) and three fallow deer (*Dama dama*) were tested for the presence of *Anaplasma marginale/A. ovis*, *A. phagocytophilum*, *Anaplasma/Ehrlichia* spp., *Babesia/Theileria* spp., *Borrelia burgdorferi* (*sensu lato*) (*s.l.*), and *Rickettsia* spp. DNA by PCR.

Results: *Anaplasma* spp. DNA was detected in 33 (43.4 %) cervids (31 red deer and two fallow deer) and in two (3.1 %) wild boars while *Theileria* spp. were found in 34 (44.7 %) cervids (32 red deer and two fallow deer) and in three (4.6 %) wild boar blood samples. Sequence analysis of *msp4* sequences identified *A. marginale*, *A. ovis*, while the analysis of *rDNA* sequence data disclosed the presence of *A. platys* and *A. phagocytophilum* and *T. capreoli* and *Theileria* sp. OT3. *Anaplasma* spp./*Theileria* spp. mixed infections were found in 17 cervids (22.4 %) and in two wild boars (3.1 %). All samples were negative for *Babesia* sp., *B. burgdorferi* (*s.l.*), *Ehrlichia* sp. or *Rickettsia* sp.

Conclusions: This is the first detection of *Anaplasma marginale*, *A. ovis*, *A. phagocytophilum*, *A. platys*, *Theileria capreoli* and *Theileria* sp. OT3 in cervids and wild boars from Portugal. Further studies concerning the potential pathogenicity of the different species of *Anaplasma* and *Theileria* infecting wild ungulates, the identification of their vector range, and their putative infectivity to domestic livestock and humans should be undertaken.

Keywords: *Anaplasma* spp., Fallow deer, PCR, Portugal, Red deer, *Theileria* spp., Tick-borne pathogens, Wild boar

Background

Wildlife can harbor a high density of ticks that can transmit several pathogens, such as bacteria, parasites and viruses. In addition to their veterinary importance, many of these tick-borne pathogens can also affect the human population as a result of their zoonotic potential. Therefore, the management of such situation calls for a One Health approach, including the increased awareness for their presence especially in sylvatic environments and areas associated with animal husbandry among veterinarians, physicians and general public [1].

Piroplasmoses in cattle is caused by tick-borne protozoan parasites comprising several *Theileria* and *Babesia* species. These diseases are a serious health problem, being responsible for important economic losses to the cattle industry. In Europe, infections with different *Theileria* spp. [*Theileria* sp. OT3, *T. capreoli* (formerly *Theileria* sp. 3185/02), *Theileria* sp. ZS OT4, *T. ovis*] and *Babesia* spp. [including, among others, *B. bigemina*, *B. capreoli*, *B. divergens* and *B. venatorum* (formerly *Babesia* sp. EU1)] have been reported in cervids. These include fallow deer (*Dama dama*), red deer (*Cervus elaphus*) and

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roe deer (*Capreolus capreolus*) [2–5], while *Theileria* sp. and *B. bigemina* were detected in wild boars (*Sus scrofa*) [5]. In Portugal, the main pathogenic piroplasm species reported in cattle is *Theileria annulata*, although others, including *T. buffeli* and *T. orientalis*, considered as being moderately pathogenic or benign, are also present [6, 7]. In addition, several pathogenic species of *Babesia* (*B. bovis*, *B. divergens* and *B. bigemina*) have also been reported in cattle from central and southern Portugal [6, 7]. Further, human babesiosis caused by *B. divergens*, *B. microti* or *B. venatorum* have been reported in several European countries [8], including one fatal case due to *B. divergens* in Portugal [9].

Anaplasmoses, caused by bacteria of the genus *Anaplasma*, known for a long time in veterinary medicine, are also considered as emerging human diseases, and are frequently associated with infection with *Anaplasma phagocytophilum* [10]. This bacterium, which is the causative agent of tick-borne fever, a disease of important negative economic impact to European animal husbandry (involving domestic ruminants), also causes human granulocytic anaplasmosis. Wild ruminants are one of its main reservoirs [11] while the role of wild boars in its natural cycle is still contradictory [12]. Other *Anaplasma* spp. such as *A. marginale* and *A. ovis* have also been detected in European cervids [13]. In Portugal, antibodies reactive to *A. phagocytophilum* antigens were detected in humans and other mammals [14], while *A. marginale* and *A. ovis* were detected in cattle [15] and in sheep [16], respectively.

Among the diseases caused by tick-borne pathogens, Lyme borreliosis caused by spirochetes of the *Borrelia burgdorferi* (*sensu lato*) (*s.l.*) complex is currently the most common tick-borne disease in Europe [17]. In Portugal, its notification in humans is mandatory, but the disease is clearly underdiagnosed and underreported [17]. Wild large vertebrates seem to be frequently exposed to these bacteria, as indicated by the detection of either *Borrelia*-specific antibodies in these animals or *Borrelia* DNA in engorged ticks collected from them [18, 19]. Finally, several tick-borne *Rickettsia* spp. associated with human infections such as *Rickettsia conorii, R. slovaca* and *R. raoultii* have also been described in several European countries, including Portugal [20]. *Rickettsia* spp. (e.g. *R. helvetica*, *R. slovaca*) DNA has previously been detected in the peripheral blood [21] or in ticks removed from cervids and wild boars [4, 22], but the role these wild mammals play in the natural maintenance of these bacteria has not yet been clarified.

No information about tick-borne pathogens circulating in wild ungulates from Portugal is available, with the single exception of the recent detection of *Borrelia burgdorferi* (*s.l.*) in wild boars from northern Portugal [17]. Thus, the aim of the present study was to assess the presence of tick-transmitted bacteria and protozoa with veterinary and zoonotic importance in cervids and wild boars from the Centre and South of the country.

Methods

Animals and samples

During the hunting seasons, from December 2013 to March 2015, a total of 141 free-ranging ungulates including 73 red deer (*Cervus elaphus*), 65 wild boars (*Sus scrofa*) and 3 fallow deer (*Dama dama*) from both sexes were sampled in the districts of Castelo Branco ($n = 31$), Portalegre ($n = 16$), Lisboa ($n = 19$), Évora ($n = 15$) and Beja ($n = 60$). Animals were classified in two age categories: young (1–3 years) and adults (> 3 years). Blood samples were collected from each animal by cardiac or thoracic punctures in EDTA tubes and stored at -20 °C until DNA extraction.

Ethical approval

This study was ethically approved by the board of the Faculty of Veterinary Medicine (ULHT).

PCR amplification

A commercial kit (PCR-template Preparation kit, Roche Diagnostics GmbH, Germany) was used to extract DNA from the collected blood samples, following the manufacturer's instructions.

In order to avoid false negative results due to PCR inhibition, and so as to validate the efficiency of the DNA extraction, the modified vertebrate-universal *cyt-b* specific primers (cytB1-F and cytB2-R) were used to amplify a 350 bp segment of the host mitochondrial *cytochrome b* gene (*cyt-b*) [23]. PCR amplifications were performed in a 25 μl final volume containing 12.5 μl of NZYTaq 2x Green Master Mix (Nyztech, Portugal), 1 μl of each primer (10 pmol) and 2 μl of template DNA.

Detection of *Anaplasma*/*Ehrlichia* spp., *A. marginale*/*A. ovis*, *A. phagocytophilum*, *Babesia*/*Theileria* spp., *B. burgdorferi* (*s.l.*) and *Rickettsia* spp. DNA in blood samples was assessed by PCR, according to previously described protocols (Table 1).

PCR amplifications were performed in a final volume of 25 μl using NZYTaq 2× Green Master Mix, 3 μl of the prepared DNA extracts and 10 pmol of each primer. In all amplifications, positive (containing genomic DNA of the targeted microrganism) and negative (without DNA) controls, were included. PCR amplifications were carried out in a Thermo Electron Corporation® Px2 Termal Cycler (VWR, USA) and the obtained PCR products visualized under UV illumination after electrophoresis on 1.5 % agarose gels stained with Greensafe premium® (Nzytech, Portugal) using a 100 bp DNA ladder as a molecular-weight size marker (Nzytech, Portugal).

Table 1 Sequences of the oligonucleotide primers used

Pathogen	Target gene		Oligonucleotide sequences (5'-3')		Amplicon size (bp)	Reference
			Forward	Reverse		
Anaplasma spp./*Ehrlichia* spp.	*16S rRNA*		GGTACCYACAGAAGAAGTCC	TAGCACTCATCGTTTACAGC	345	[38]
	groEL		ACTGATGGTATGCARTTTGAYCG	TCTTTRCGTTCYTTMACYTCAACTTC	600	[39]
Anaplasma marginale/*A. centrale*/*A. ovis*	*msp4*		GGGAGCTCCTATGAATTACAGAGAATTGTTTAC	CCGGATCCTTAGCTGAACAGGAATCTTGC	851	[40]
Anaplasma phagocytophilum	*msp4*		ATGAATTACAGAGAATTGCTTGTAGG	TTAATTGAAAGCAAATCTTGCTCCTATG	849	[13]
Babesia spp./*Theileria* spp.	*18S rRNA*		AATACCCAATCCTGACACAGGG	TTAAATACGAATGCCCCCAAC	400	[38]
Borrelia burgdorferi (*sensu lato*)	*ITS 5S-23S rRNA*	Outer primmers	ACCATAGACTCTTATTACTTTGAC	TAAGCTGACTAATACTAATTACCC	380	[41]
		Inner primers	ACCATAGACTCTTATTACTTTGACCA	GAGAGTAGGTTATTGCCAGGG	225	
	flaB	Outer primmers	TGGTATGGGAGTTTCTGG	TAAGCTGACTAATACTAATTACCC	774	[42]
		Inner primers	CAGACAACAGAGGGAAAT	TCAAGTCTATTTTGGAAAGCACC	604	
Rickettsia spp.	*gltA*		GCGGGCCTGCTCACGGCGG	ATTGCAAAAAGTACAGTGAACA	381	[43]

PCR products were purified from agarose gel slices with NZYGelpure® (Nzytech, Portugal) according to the manufacturer's instructions. Purified products were sent to LIGHTrun™ Sequencing Service (GATC-biotech, Germany) for direct sequencing of the obtained amplicons by Sanger's method with the same primers used for DNA amplification.

DNA sequence analyses

Species identity of the obtained sequences was assessed on the basis of the closest BLASTn match (identity ≥ 98 % using the MegaBLAST and a query cover no smaller than 96 %) with homologous sequences deposited in the GenBank database. The sequences obtained in the course of this work were deposited at DNA Data Bank of Japan (DDBJ) (http://www.DDBJ.nig.ac.jp).

Phylogenetic relationships were inferred from nucleotide sequence alignments produced with the MAFFT multiple alignment program using a combination of the G-INS-i alignment option [24]. Phylogenetic tree construction was carried out using a Maximum Likelihood (ML) approach, using the Kimura's 2-P (K2P) evolutionary model, and assuming a Γ distributed substitution rates among sites, as indicated by Mega6 [25] on the basis of the Akaike information criterion. Alternatively, an empirically defined model (GTR + Γ + I) was also used. The topological robustness of the obtained trees was assessed by bootstrapping, using 1000 resampling of the original alignment data. The final trees were manipulated for display using FigTree v.1.2.2. (available at http://tree.bio.ed.ac.uk/software/figtree/).

Statistical analysis

Percentages of positive samples for *Anaplasma* spp. and *Theileria* spp. regarding the independent variables and categories were compared by the Chi-square or Fisher's exact tests. A P-value ≤ 0.05 was considered as statistically significant. Exact binomial 95 % confidence intervals (CI) were defined for the proportions. Analyses were performed with Epi Info™ 7.1.5.2 software for Centers for Disease and Prevention.

Results

A 350 bp fragment of the host mitochondrial *cyt-b* gene was amplified in all DNA blood samples.

Anaplasma spp. DNA was detected in 33 (43.4 % CI: 32.1–55.3 %) cervids (31 red deer and two fallow deer) and in two (3.1 % CI: 0.4–10.7 %) wild boars using a set of general primers that target *16S rDNA*. Seventeen sequences obtained from red deer (accession numbers: LC126854, LC126858-9, LC126863-5, LC126867-9, LC126871, LC126873, LC126875, LC126878, LC126879 and LC126881-3) and two from wild boars (accession numbers: LC126885-6) showed 99–100 % identity with *A. platys* previously described in dogs from Portugal (LC018182-3; [26]), Argentina (JX261979; [27]) and in a goat from Cyprus (EU090182; [28]). Further, eight sequences obtained from red deer (LC126855-6,

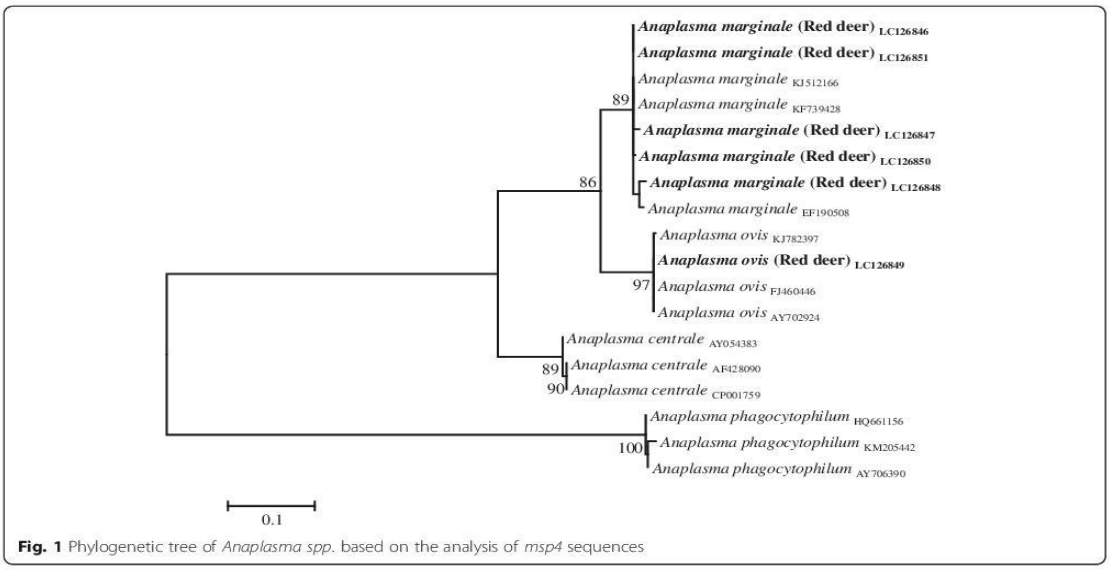

Fig. 1 Phylogenetic tree of *Anaplasma spp.* based on the analysis of *msp4* sequences

LC126860-2, LC126870, LC126876 and LC126884) showed 99 % identity with *A. phagocytophilum* described in Swedish moose (KC800983; [29]) and in black-striped field mice (*Apodemus agrarius*) in South Korea (KR611719). Six sequences isolated from red deer (LC126857, LC126866, LC126872, LC126874 and LC126880) and two isolated from fallow deer (LC126852-3) showed 100 % identity with *A. marginale* (KC335218, KC335223) described in cows and with *A. ovis* described in sheep (KC335231) and goats (KC335225) from Italy [30] as well as with *A. centrale* described in goats from China (KP062964, KP062966; [31]).

Sequencing of the *msp4* gene amplified from the samples where the presence of *A. centrale/A.marginale/A. ovis* DNA had been detected with the *16S rDNA* primers, confirmed the presence of *A. marginale* in five (6.6 % CI: 2.2–14.7 %) red deer from the Castelo Branco, Portalegre and Beja districts and *A. ovis* in one (1.3 % CI: 0–7.1 %) red deer from Beja. Attempts to amplify *msp4* sequences from the two fallow deer failed. The obtained *msp4* sequence data, along with related sequences obtained from Genbank, were subjected to phylogenetic analyses (Fig. 1).

PCR reactions prepared using either *groEL* or *A. phagocytophilum* species-specific primers revealed reproducibly negative amplification results. On the contrary, *Theileria* spp. were found in 34 (44.7 % CI: 33.3–56.6 %) cervids (32 red deer and two fallow deer) and in three (4.6 % CI: 1.0–12.9 %) wild boar samples, using primers targeting the *18S rRNA* gene. Blast analysis showed that the sequences obtained from red deer (accession numbers: LC131069-100) and wild boars (LC131101-3) presented 98–99 % identity to *T. capreoli* (KJ188207-8) described in Sika deer from China [32] while the two sequences obtained from fallow deer (LC131067-8) showed a high identity (98–99 %) to the *Theileria* sp. OT3 (Genbank: KF470868) described in sheep from China [33]. The phylogenetical analysis of the obtained *18S rDNA* sequences along with the related sequences from GenBank corroborated the Blast identification (Fig. 2).

Anaplasma spp./*Theileria* spp. co-infections were found in 17 cervids (22.4 % CI: 13.6–33.4 %). Of these, eight red deer were co-infected with *A. platys* and *T. capreoli*, four with *A. marginale* and *T. capreoli*, two with *A. phagocytophilum* and *T. capreoli*, one with *A. ovis* and *T. capreoli*, while two fallow deer were co-infected with *Anaplasma* sp. and *Theileria* sp. OT3. Two wild boars (3.1 % CI: 0.4–10.7 %) were co-infected with *A. platys* and *T. capreoli*.

The frequency of *Anaplasma* infection was significantly higher ($P = 0.019$) in red deer from the Castelo Branco district than from Beja (Table 2). All the blood samples taken from wild boars with positive PCR amplification results to *T. capreoli* or *A. platys* were from the Beja district.

Fig. 2 Phylogenetic tree of *Theileria* spp. based on *18S rRNA* gene sequences

Table 2 Prevalence of tick-borne pathogens as detected by PCR in 76 cervids and 65 wild boars from Centre and southern Portugal

Idependent variable/category	*Cervus elaphus & Dama dama*					*Sus scrofa*				
	No. (%)	*Anaplasma* spp.		*Theileria* spp.		No. (%)	*Anaplasma* spp.		*Theileria* spp.	
		% of positive	95 % CI	% of positive	95 % CI		% of positive	95 % CI	% of positive	95 % CI
District	76	ND*		ND*		65	ND*		ND*	
Castelo Branco	27 (35.5)	59.3[a]	38.8–77.6	44.4	25.5–64.7	4 (6.2)	0	0–60.2	0.0	0.0–60.2
Portalegre	1 (1.3)	100	2.5–100	100	2.5–100	15 (23.1)	0	0–21.8	0.0	0.0–21.8
Lisboa	3 (3.9)	66.7	9.4–99.2	66.7	9.4–99.2	16 (24.6)	0	0–20.6	0.0	0.0–20.6
Évora	–	–	–	–	–	15 (23.1)	0	0–21.8	0.0	0.0–21.8
Beja	45 (59.2)	31.1[a]	18.2–46.7	42.2	27.7–57.9	15 (23.1)	13.3	1.66–40.5	20.0	4.3–48.1
Age	61	$P = 0.309$		$P = 0.607$[c]		62	$P = 1$		$P = 0.545$	
Adult	50 (82.0)	40.0	26.4–54.8	46.0	31.8–60.7	42 (67.7)	4.8	0.6–16.2	7.1	1.5–19.5
Young	11 (18.0)	18.2	2.3–51.8	54.5	23.4–83.3	20 (32.3)	0	0–16.8	0.0	0.0–16.8
Gender	69	$P = 0.618$[b]		$P = 0.025$[d]		63	$P = 0.493$		$P = 1$	
Female	37 (53.6)	37.8	22.5–55.2	32.4	18.0–49.8	45 (71.4)	2.2	0.1–11.8	4.4	0.5–15.2
Male	32 (46.4)	43.8	26.4–62.3	59.4	40.7–76.3	18 (28.6)	5.6	0.1–27.3	5.6	0.1–27.3
Total	76	43.4	32.1–55.3	44.7	33.3–56.6	65	3.1	0.4–10.7	4.6	1.0–12.9

[a] $\chi^2 = 5.50$, $df = 1$, $P = 0.019$
[b] $\chi^2 = 0.25$, $df = 1$
[c] $\chi^2 = 0.26$, $df = 1$
[d] $\chi^2 = 5.03$, $df = 1$
ND* Statistically significant difference(s) not confirmed after pairwise comparisons

None of the samples analysed revealed the presence of *Babesia* sp., *B. burgdorferi* (*s.l.*), *Ehrlichia* sp. or *Rickettsia* sp.

Discussion

Concern about the role of wildlife in the natural maintenance transmission of tick-borne pathogens is increasing, especially in areas where free-ranging animals regularly interact with domestic livestock and humans [5, 34].

This study, which aimed at the detection of tick-borne bacteria and protozoa of veterinary and zoonotic importance in cervids and wild boars, disclosed, to our knowledge, the first evidence for the circulation of *Anaplasma* spp. and *Theileria* spp. among red deer, fallow deer and wild boars in central/southern Portugal. In this study, *Anaplasma* spp. infections were detected in the three wild ungulate species analysed as revealed by the amplification of *16S rDNA* sequences using genus-specific primers. *Anaplasma platys* causes canine cyclic thrombocytopenia and is presumably transmitted by ticks of the *Rhipicephalus sanguineus* group. As *A. platys* DNA has previously been reported in dogs [26], ticks [35] and red foxes [36] from Portugal, its detection in the red deer and wild boars sampled herein indicates that these animals are also exposed to the bacterium. However, the ability of *A. platys* to cause disease in these animals has not been established yet.

As wild cervids are considered one of the main reservoirs of *A. phagocytophilum* [11], the detection of this bacterium in eight red deer blood samples using *16S rDNA* primers it is not entirely surprising, especially when it is known that the pathogen is circulating in different Portuguese vertebrate hosts, as well as in *Ixodes ricinus,* its most frequently associated vector [14]. However, the absence of detection of *msp4* specific sequences may indicate the circulation of divergent *A. phagocytophilum* variants among Portuguese red deer different from the ones previously reported [37]. In any case, this issue deserves future clarification. Furthermore, the presence of *A. ovis* and *A. marginale* in red deer was confirmed by *msp4* phylogenetic analysis, confirming the susceptibility of this cervid to the agents responsible for bovine and ovine anaplasmoses [13]. Both pathogens have been reported in cattle from the Alentejo region (which includes the Évora and Beja districts [15]), and in sheep raised throughout the country [16].

The occurrence of *Theileria* spp. infections in European cervids is well documented [2–5]. In the present study, and for the first time in Portugal, *T. capreoli* and *Theileria* sp. OT3 *msp4* sequences were amplified from red deer and fallow deer samples, respectively, corroborating previous data from northern Spain [3]. The overall prevalence of *Babesia* spp. and *Theileria* spp. infections previously reported in cattle from the central and southern regions of Portugal ranged from 23.1 % [7] to 74.7 % [6], respectively, with *T. annulata* and *T. buffeli* being the most commonly detected species and *B. bigemina*, *B. bovis* and *B. divergens* being the least frequently found. As none of the *Theileria* and *Babesia* species known to circulate in the Portuguese cattle were detected in the present study, it seems that the tested wild ungulate species might not play a significant role in their transmission, at least in the regions where samples were collected. Furthermore, although deer have been previously appointed as the source for ovine infection with *Theileria* sp. OT3 [3], no data is yet available regarding the circulation of piroplasmids in small ruminants from Portugal.

Despite the fact that *B. burgdorferi* (*s.l.*) and *Rickettsia* spp. have already been detected in ticks and/or in the blood collected from cervids and wild boars [4, 17, 19, 22], their presence was not revealed in any of the samples analysed in the present study. This observation supports the hypothesis that wild ungulates, at least in the studied areas, are not pivotal players in the natural maintenance cycles of these bacteria, as previously reported [4, 21].

As large wildlife are important to maintain tick populations, and since ticks may become infected with several pathogens during their life cycle, the detection of *Anaplasma* spp./*Theileria* spp. co-infections in the present study is not surprising, and falls in line with previously published observations in wild ungulates [4]. The interaction of different pathogens within the vertebrate host might lead to increased susceptibility to other infections as well as a modification of the pathogenesis of each microorganism with profound consequences for disease management programs and wildlife conservation [4].

Conclusions

The present study provides information regarding the presence of *Anaplasma marginale, A. ovis, A. phagocytophilum, A. platys, Theileria capreoli* and *Theileria* sp. OT3 in cervids and wild boars from central and southern Portugal. Further studies concerning the potential pathogenicity of the different *Anaplasma* and *Theileria* species infecting wild ungulates, the identification of their vector range, and their infectivity to domestic livestock and humans should be undertaken.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AP performed DNA extraction, PCR assays and data analysis; RP performed DNA sequence analysis and revised the manuscript; MN performed *B. burgdorferi* s.l. nested-PCRs; AC collected blood samples and animal data; MLV and LC reviewed the manuscript; CM planned, designed and supervised the study, and wrote the manuscript; All authors read and approved the final manuscript.

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Short Communication

First Detection of *Borrelia burgdorferi* sensu lato DNA in Serum of the Wild Boar (*Sus scrofa*) in Northern Portugal by Nested-PCR

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Abstract: Lyme borreliosis is the most common tick-borne zoonosis in the northern hemisphere. Several vertebrates are crucial in the epidemiological cycle of *Borrelia burgdorferi* sensu lato, but the role of wild boar as a reservoir is still unknown. Sera were collected from 90 wild boars shot in the Trás-os-Montes region, Northern Portugal (hunting season 2011/2012). In this study, *Borrelia* DNA was detected for the first time by nested-PCR in three different sera, suggesting that the wild boar may be a potential reservoir for this spirochete. Sequencing results show 100% similarity with *Borrelia afzelii*. Further studies are needed to evaluate the public health risks associated with boar hunting.

Keywords: *Borrelia burgdorferi* sensu lato, wild boar, PCR, *fla* gene, Northern Portugal, Lyme borreliosis, zoonosis, One Health

Lyme borreliosis (LB) is a zoonosis caused by spirochetes of the *Borrelia burgdorferi* sensu lato (s.l.) complex and it is currently the most common tick-borne disease in Europe and North America (Rizzoli et al. 2011; EUCALB 2014), with *Ixodes ricinus* being the main vector (Gern 2009). Furthermore, due to its emerging infectious disease status, LB is specifically targeted by the European Center for Disease Control and Prevention (ECDC 2014) and should be regarded from a "One Health" perspective. In Portugal, notification of LB in humans is mandatory, but the disease is still underdiagnosed and underreported (Couceiro et al. 2003; Lopes de Carvalho and Núncio 2006).

David de Morais et al. (1989) described the first human clinical case of LB in Portugal. Few years later, occurred the very first isolation of *B. lusitaniae*, initially from an *I. ricinus* tick (Núncio et al. 1993; Le Fleche et al. 1997), and later on from a skin biopsy of a Portuguese patient

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(Collares-Pereira et al. 2004). Many other isolations of this and other species of the *B. burgdorferi* s.l. complex have been made from the vector and from animal hosts (e.g., *Apodemus sylvaticus*, *Turdus merula*) (De Michelis et al. 2000; Baptista et al. 2004; Lopes de Carvalho et al. 2010; Norte et al. 2012).

Human infection is higher in areas where the disease is endemic, especially in forested regions where the vector is abundant (Lindgren and Jaenson 2006). Hunters and gamekeepers are two hunting-related occupations flagged as high risk for infection (Christiann et al. 1997; Hubalek 2009) and the overall population density of game species like deer or wild boar (*Sus scrofa*) can in some cases be correlated with LB risk (Zeman and Januska 1999).

Several studies have suggested a potential role of the wild boar in the epidemiological cycle of LB, either through the detection of antibodies against *B. burgdorferi* s.l. in wild boar sera (Juricova and Hubalek 2009) or through the detection of both wild boar DNA and *Borrelia* DNA in the blood meal of ticks (Estrada-Peña et al. 2005; Móran Cadenas et al. 2007; Wodecka et al. 2014). Detection of *Borrelia* DNA in wild boar and its parasitizing ticks was already attempted by Silaghi et al. (2014), but borrelial DNA was not found in any of the analyzed wild boar samples (blood and tissues), only in one of the analyzed *I. ricinus* ticks. Despite all these efforts, the role of the wild boar remains largely unknown.

The goal of this study was to assess the relevance of wild boar as potential reservoir host in the eco-epidemiological cycle of LB by analyzing the presence of *B. burgdorferi* s.l. DNA in its sera.

Blood samples were collected from 90 wild boars shot during the hunting season of 2011/2012 in the Trás-os-Montes region, Northern Portugal (districts of Bragança and Vila Real), centrifuged for serum extraction and stored at −20°C until further analysis.

Additional animal information was also collected: county and district of provenience, sex and age. Age estimation was based on tooth eruption patterns and according three classes: juveniles (7–12 months), subadults (12–24 months), and adults (>24 months) (Sáenz de Buruaga et al. 1991).

Detection of *Borrelia* DNA in sera of wild boar was accomplished using a nested-PCR protocol that targets the flagellin-encoding gene *fla* (outer primers: 132f/905r; inner primers: 220f/823r) (Wodecka 2007, as cited in Wodecka et al. 2010), a molecular target chosen in other studies (Skotarczak et al. 2008; Wodecka et al. 2009, 2014; Kiewra and Zaleśny 2013) and considered genus specific for *Borrelia* (Situm et al. 2000). PCR assays targeting the *fla* gene have a significantly higher sensitivity in relation to other DNA markers both for detection of *B. burgdorferi* s.l. in *I. ricinus* (Wodecka et al. 2010) and in clinical samples (Situm et al. 2000).

DNA extraction from sera was performed using Gentra Puregene Blood Kit®, (QIAGEN®) according to the manufacturer's instructions. The reaction mixture (25 μl volume) contained 1 U BioTaq™ DNA Polymerase, 1× NH_4 reaction buffer, 1.5 mM $MgCl_2$, 0.25 mM of each deoxynucleotide triphosphate (Bioline®) and 1 μM of each primer of outer and inner pairs of primers (Eurofins MWG Operon, Germany). DNA template used in the first amplification was a 2.5 μl suspension of the extraction product and 1 μl of the first amplification product was used as template for the second amplification. Ultrapure water was used as negative control and DNA from a culture of *B. burgdorferi* sensu stricto B31 strain was used as positive control. To insure no inhibitory compounds affected the nested-PCR reaction, a conventional PCR targeting the mammalian *β*-actin housekeeping gene (primers F_Actin/R_Actin) was used as an internal control (Costa et al. 2013).

Initialization cycle took place at 94°C for 10 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing for 45 s at 50°C for the outer primers (first PCR) or 54°C for the inner primers (second PCR) and elongation at 72°C for 1 min. The final elongation cycle ran at 72°C for 7 min. PCR products were subjected to agarose gel electrophoresis and sequenced at Stab Vida® (Lisbon, Portugal). The nucleotide sequences were submitted to GenBank under the accession number KJ810661 and compared with available *B. burgdorferi* s.l. reference strains using BLASTN 2.2.29+ (Camacho et al. 2008).

The wild boar population analyzed encompassed 36 adults, 30 subadults, and 24 juveniles, represented by 61 females and 29 males. Molecular analysis of the sera confirmed the presence of *Borrelia* DNA in 3 of the 90 (3.3%) analyzed animals (Fig. 1a, lanes no. 2, 4 and 5). Positive samples were collected in the Vila Real district: a subadult female (J26) from Montalegre County, and from Alijó County an adult male (J30) and female (J31) (Fig. 1b). Samples J26 and J31 yielded gene sequences that matched 100% to that of *Borrelia afzelii* strain LO-3.9 (e.g., GenBank Accession KF990318). Sequencing results for sample J30 (Fig. 1a, lane 4) were inconclusive due to low quality of the sequences obtained.

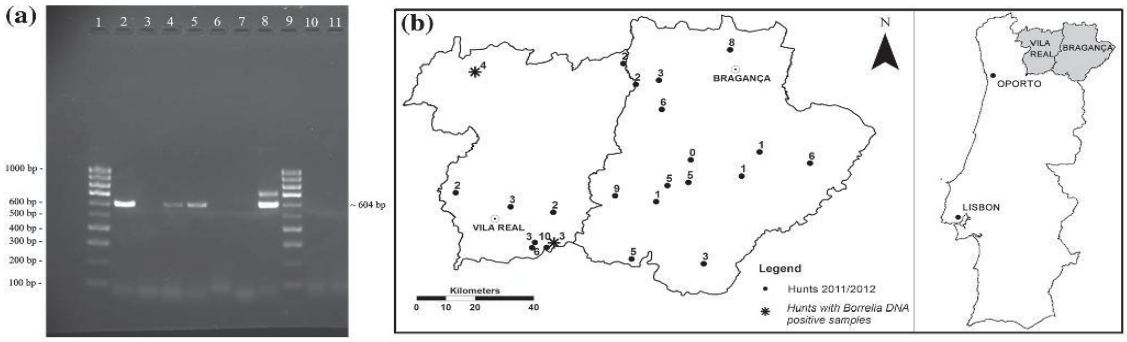

Figure 1. **a** DNA amplification results of wild boar serum samples obtained by nested-PCR analysis targeting the *fla* gene; negative extraction controls (*lanes 6* and *7*); positive control (*lane 8*); negative control for first PCR amplification (*lane 10*); negative control for second PCR amplification (*lane 11*). Samples J26, J30, and J31 are positive for *B. burgdorferi* s.l. DNA (*lanes 2*, *4*, and *5*, respectively). 100–1000 bp DNA ladder (*lanes 1* and *9*); **b** Geographical distribution of the hunts (*filled circle*) attended during the 2011/2012 wild boar hunting season in the Trás-os-Montes region (Northern Portugal) and the number of wild boars shot in each hunt. Marked with an *asterisk* are the hunts where the samples positive for *Borrelia* DNA were collected.

To our knowledge, this is the first time that *Borrelia* DNA is amplified from wild boar serum, providing a significant step toward the confirmation of a previously suspected but unproven notion that the wild boar could be a potential reservoir host for *B. burgdorferi* s.l. (Estrada-Peña et al. 2005; Móran Cadenas et al. 2007; Juricova and Hubalek 2009).

The positive identification of *B. afzelii* in wild boar is an important epidemiological finding since this genospecies is usually associated with small mammals, particularly rodents, and previously detected in Portugal in questing ticks (Kurtenbach et al. 2001; Baptista et al. 2004). This hypothetical association between *B. afzelii* and the wild boar has already been suggested in previous studies (Estrada-Peña et al. 2005; Móran Cadenas et al. 2007; Wodecka et al. 2014), where DNA of both was detected simultaneously in ticks. Additionally, the presence of antibodies to *B. burgdorferi* s.l. in wild boar sera was detected in European countries (Doby et al. 1991; Juricova et al. 1996, 2000; Juricova and Hubalek 2009). Nevertheless, and independently of it ever being confirmed or not as a competent reservoir host for *B. afzelii*, wild boar still provides a blood source for co-feeding transmission from infected to uninfected ticks (Richter et al. 2002; Gern 2009).

Studies have shown that dogs are competent reservoirs for *B. burgdorferi* (Mather et al. 1994). Consequently, ticks that feed on infected dogs are likely to become infected as well. Wild boar hunting dogs in particular may function as a link between the wild and the domestic *Borrelia* transmission cycle, by carrying ixodids into the hunters' households, exposing them and their families to potentially infected ticks.

The results of this study serve as an indicator of the presence of this pathogen in two different areas of the Trás-os-Montes region, Northern Portugal. The circulation of *B. afzelii* in these areas may represent an important infection risk, namely for occupational groups connected to hunting activities.

Further epidemiological studies concerning borreliosis are required for proper infection risk assessment in the Trás-os-Montes region. This evaluation is essential to the development of effective disease control and prevention strategies, rooted in a multidisciplinary approach that encompasses both human and animal health.

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4.3 - Bacterial and protozoal agents of canine vector-borne diseases in the blood of domestic and stray dogs from southern Portugal

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Bacterial and protozoal agents of canine vector-borne diseases in the blood of domestic and stray dogs from southern Portugal

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Abstract

Background: The so-called canine vector-borne diseases (CVBD) are caused by a wide range of pathogens transmitted by arthropods. In addition to their veterinary importance, many of these canine vector-borne pathogens can also affect the human population due to their zoonotic potential, a situation that requires a One Health approach. As the prevalence of vector-borne pathogens in cats from southern Portugal has been recently evaluated, the aim of the present study was to assess if the same agents were present in dogs living in the same area, and to assess positivity-associated risk factors.

Methods: One thousand and ten dogs (521 domestic and 489 stray) from veterinary medical centres and animal shelters in southern Portugal were enrolled. *Anaplasma* spp./*Ehrlichia* spp., *Bartonella* spp., *Borrelia burgdorferi* sensu lato, *Babesia* spp., *Hepatozoon* spp. and *Leishmania infantum* infections were evaluated by polymerase chain reaction (PCR) assays in blood samples.

Results: Sixty-eight (6.7%) dogs were PCR-positive to at least one of the tested CVBD agent species, genera or complex, including one dog found positive to two different genera. Nineteen (1.9%) dogs were positive to *Anaplasma* spp./*Ehrlichia* spp., eight (0.8%) to *B. burgdorferi* s.l., 31 (3.1%) to *Hepatozoon* spp. and 11 (1.1%) to *L. infantum*. *Anaplasma platys*, *Ehrlichia canis*, *B. burgdorferis*.l. and *Hepatozoon canis* were identified by DNA sequencing, including one animal confirmed with both *A. platys* and *H. canis*. Furthermore, *Wolbachia* spp. was amplified in blood from four dogs. None of the tested dogs was positive by PCR for *Bartonella* spp. or *Babesia* spp.

Conclusions: The molecular identification of CVBD agents in southern Portugal, some of them with zoonotic concern, reinforces the importance to alert the veterinary community, owners and public health authorities to prevent the risk of transmission of vector-borne pathogens among dogs and to other vertebrate hosts including humans. The prevalence of the selected pathogens was lower than that previously found in cats from the same region, probably because veterinarians and owners are more aware of them in the canine population and control measures are used more often.

Keywords: Dogs, Canine vector-borne diseases, Bacteria, Protozoa, Portugal, Polymerase chain reaction

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Background

Canine vector-borne diseases (CVBD) comprise a group of globally distributed and spreading illnesses that are caused by a wide range of pathogens transmitted by arthropods [1-4]. In addition to their veterinary importance, many of these canine vector-borne pathogens can also affect the human population due to their zoonotic potential, a situation that requires a One Health approach [5,6].

Anaplasma phagocytophilum and *Anaplasma platys* cause canine granulocytic anaplasmosis and infectious canine cyclic thrombocytopenia, respectively. Both agents can infect a range of domestic and wild vertebrate hosts, including dogs and humans [7-10]. *A. phagocytophilum* is transmitted by ticks of the genus *Ixodes* and *A. platys* presumably by the *Rhipicephalus sanguineus* ticks. In Portugal *A. platys* DNA has been detected in clinically suspect dogs living in the north and south of Portugal [11,12], while the overall national seroprevalence of *Anaplasma* spp. has ranged from 4.5% in apparently healthy to 9.2% in clinically suspect dogs [3]. *Ehrlichia canis* (transmitted by *R. sanguineus*) is a causative agent of acute or chronic canine monocytic ehrlichiosis. *E. canis* has been molecularly detected in dogs from the north [12,13] and from the south of Portugal [14]. Seroprevalence at the national level ranged from 4.1% in apparently healthy dogs to 16.4% in animals clinically suspected of a CVBD [3].

Seven *Bartonella* species transmitted by several arthropod vectors, including fleas and *Ixodes* spp. ticks, have been implicated as canine pathogens [15]. To date, no dog with *Bartonella* spp. infection has been reported in Portugal. Spirochetes belonging to the *Borrelia burgdorferi* sensu lato complex are the agents of Lyme borreliosis. In Europe, *B. burgdorferi* s.l. is mainly transmitted by *I. ricinus* [16]. Though few infected dogs show similar clinical signs, most of them are subclinical hosts [17] and can be sentinels for this infection. In Portugal, seropositivity to *B. burgdorferi* s.l. has ranged from 0.2% in apparently healthy dogs to 0.5% in clinical suspected animals in a country-wide investigation [3].

Canine piroplasmosis or babesiosis, mainly caused by several *Babesia* spp. haemoparasites, is a protozoal tick-borne disease with worldwide distribution [18]. *Babesia canis* (transmitted by *Dermacentor reticulatus*), *Babesia vogeli* (transmitted by *R. sanguineus*) and the *Babesia microti*-like piroplasm (syn. *Theileria annae*) were molecularly confirmed for the first time in Portugal in dogs from the north of the country [19,20]. Canine hepatozoonosis caused by the protozoan *Hepatozoon canis* transmitted by the ingestion of *R. sanguineus* is a common infection of dogs from the Old World [21]. *H. canis* has already been molecularly detected in dogs from the north [13,22] and from the south of Portugal [23]. Canine leishmaniosis (CanL), a zoonotic disease endemic in southern Europe is caused by the protozoan *L. infantum* transmitted by *Phlebotomus* spp. sand flies [24]. CanL is endemic in Portugal, with an overall national seroprevalence of 6.3% [25].

As the prevalence of vector-borne pathogens in cats from southern Portugal was recently evaluated [26], the aim of the present study was to assess if the same agents with veterinary and zoonotic importance were present in dogs living in the same region, and to assess positivity-associated risk factors.

Methods

Animals and samples

From December 2011 to April 2014, a total of 1,010 dogs (521 domestic and 489 stray), from veterinary medical centres and animal shelters in southern Portugal, were studied (Table 1). Animals were from the districts of Lisbon (n = 305), Setúbal (n = 453, which include 24 dogs from the contiguous districts of Évora and Beja) and Faro (n = 252).

Domestic dogs were randomly included after owners' informed consent. Consent for enrolment of stray dogs was obtained from the person in charge of each shelter. Out of the 489 stray animals, 457 were sheltered for adoption, and 32 others were captured and euthanized in the scope of official animal control programs.

Whole blood samples (1–2 ml) were collected by cephalic or jugular venipuncture and spotted onto filter paper for DNA extraction. Samples were dried at room temperature and kept at 4ºC until tested. Whenever available, data on the region, breed, gender, age, living conditions, use of acaricides/insecticides and clinical status (presence or absence of signs compatible with a CVBD) were registered for each dog (Table 1). Clinical signs comprised anorexia, muscular atrophy, dermatological manifestations, epistaxis, exercise intolerance, fever, gastrointestinal alterations, lameness, lethargy, lymphadenopathy, onychogryphosis, ocular manifestations, pale mucous or weight loss.

This study was ethically approved by the boards of the IHMT-UNL and of the Faculty of Veterinary Medicine (ULHT) as complying with the Portuguese legislation for the protection of animals (Law no. 92/1995).

PCR amplification and DNA sequencing

A commercial kit (Kit Citogene®, Citomed, Portugal) was used to extract DNA from blood on filter paper. Four discs of filter paper (4 mm in diameter each) were incubated with lysis buffer (150 μl) and 1.5 μl of proteinase K (20 mg/ml). Further DNA extraction followed the kit manufacturer's instructions.

Positivity to *Anaplasma* spp./*Ehrlichia* spp., *Bartonella* spp., *B. burgdorferi* s.l., *Babesia* spp., *Hepatozoon* spp. and *L. infantum* DNA in blood samples was tested by PCR according to previously described protocols (Table 2). PCR amplifications were performed in a 25 μl final volume

Table 1 Prevalence of vector-borne pathogen species, gender or complex as detected by PCR in 1,010 dogsfrom southern Portugal

Variable//category	N° of characterized dogs (%)	N° of positive dogs (%)				
		Anaplasma/Ehrlichia	*B. burgdorferi* s.l.	*Hepatozoon*	*L. infantum*	≥1 pathogen
Region	1,010					
Lisboa	305 (30.2)	1 (0.3)[a]	1 (0.3)	2 (0.7)[a]	7 (2.3)	11 (3.6)[a]
Setúbal	453 (44.9)	2 (0.4)[b]	3 (0.7)	10 (2.2)[b]	3 (0.7)	17 (3.8)[b]
Algarve	252 (25.0)	16 (6.3)[a,b]	4 (1.6)	19 (7.5)[a,b]	1 (0.4)	40 (15.9)[a,b]
Breed	793					
Defined	344 (43.4)	4 (1.2)	3 (0.9)	8 (2.3)	6 (1.7)	21 (6.1)
Mongrel	449 (56.6)	12 (2.7)	5 (1.1)	18 (4.0)	4 (0.9)	39 (8.7)
Gender	1,004					
Female	504 (50.2)	9 (1.8)	5 (1.0)	15 (3.0)	4 (0.8)	33 (6.5)
Male	500 (49.8)	9 (1.8)	3 (0.6)	14 (2.8)	7 (1.4)	33 (6.6)
Age (months)	938					
[1-11]	73 (7.8)	3 (4.1)	2 (2.7)	0 (0.0)	0 (0.0)	5 (6.8)
[12–83]	576 (61.4)	10 (1.7)	4 (0.7)	15 (2.6)	7 (1.2)	36 (6.3)
[84–228]	289 (30.8)	3 (1.0)	1 (0.3)	7 (2.4)	3 (1.0)	14 (4.8)
Lifestyle	1,010					
Domestic	521 (51.6)	15 (2.9)[a]	6 (1.2)	19 (3.6)	6 (1.2)	45 (8.6)[a]
Stray	489 (48.4)	4 (0.8)[a]	2 (0.4)	12 (2.5)	5 (1.0)	23 (4.7)[a]
Housing	852					
Indoors	63 (7.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)[a,b]
Mixed	182 (21.4)	5 (2.7)	3 (1.6)	11 (6.0)	0 (0.0)	19 (10.4)[a]
Outdoors	607 (71.2)	11 (1.8)	4 (0.7)	18 (3.0)	5 (0.8)	38 (6.3)[b]
Acaricides-insecticides	963					
Yes	448 (46.5)	5 (1.5)	4 (0.8)	10 (2.2)	6 (1.3)	25 (5.6)
No	515 (53.5)	12 (2.3)	4 (0.8)	19 (3.7)	4 (0.8)	38 (7.4)
Clinical status	906					
Non-suspect	700 (77.3)	12 (1.7)	5 (0.7)	26 (3.7)	7 (1.0)	49 (7.0)
Suspect	206 (22.7)	6 (2.9)	2 (1.0)	3 (1.5)	3 (1.5)	14 (6.8)
Total	1,010	19 (1.9)	8 (0.8)	31 (3.1)	11 (1.1)	68 (6.7)

[a,b]Statistically significant difference for the same agent between categories of the same variable ($p < 0.05$).

containing 12.5 μl of NZYTaq 2x Green Master Mix (Nyztech, Portugal), 1 μl of each primer (10 pmol) and 3 μl of DNA template. In all amplifications a positive control containing genomic target DNA and a negative control without DNA were included. The reaction mixtures were cycled in a Thermo Electron Corporation® Px2 Termal Cycler (VWR, USA). PCR products were visualized under UV illumination after electrophoresis migration on a 1.5% gel agarose stained with GreenSafe Premium® (Nzytech), using a 100 bp DNA ladder as a marker.

PCR products were purified with a High Pure PCR Product Purification Kit (Roche®, Germany) according to the manufacturer's instructions and directly sequenced (one direction) (Stabvida®, Portugal), using the same primers as those used for the DNA amplification. Species identity of the obtained sequences was determined according to the closest BLAST match (identity ≥ 99% for the first 30 matches) to a GenBank® accession and deposited in DNA Data Bank of Japan (DDBJ) (http://www.DDBJ.nig.ac.jp).

Statistical analysis

Percentages of positivity to CVBD agents were compared by the Chi-square or Fisher's exact tests. A p value < 0.05 was considered as statistically significant. The exact binomial test was used to calculate confidence intervals for the proportions, with a 95% confidence level (CI). Analyses were performed with SPSS® 21 software for Windows and with StatLib.

Table 2 Primers sets for PCR amplification of CVBD agents

Pathogen	Primers	Product size (bp)	Reference
Anaplasma spp./*Ehrlichia* spp.	EHR16SD: 5'-GGT ACC YAC AGA AGA AGTCC-3'	345	[27]
	EHR16SR: 5'-TAG CAC TCA TCG TTT ACAGC-3'		
Bartonella spp.	325 s: 5'-CTTCAGATGATGATCCCAAGCCTTCTGGCG-3'	500-800	[28]
	1100as: 5'-GAACCGACGACCCCCTGCTTGCAAAGCA-3'		
Borrelia burgdorferi s.l.	Outer primers:	774	[29]
	132f: 5'-TGGTATGGGAGTTTCTGG-3'		
	905r: 5'-TCTGTCATTGTAGCATCTTT-3'		
	Inner primers:	604	[29]
	220f: 5'-CAGACAACAGAGGGAAAT-3'		
	823r: 5'-TCAAGTCTATTTTGGAAAGCACC-3'		
Babesia spp.	PIRO-A: 5'-AAT ACC CAA TCC TGA CACAGG G-3'	400	[27]
	PIRO-B: 5'-TTA AAT ACG AAT GCC CCCAAC-3'		
Hepatozoon spp.	HEP-F: 5'-ATA CAT GAG CAA AAT CTC AAC-3'	626-666	[30]
	HEP-R: 5'-CTT ATT ATT CCA TGC TGC AG-3'		
Leishmania infantum	MC1: 5'-GTTAGCCGATGGTGGTCTTG-3'	447	[31]
	MC2: 5'-CACCCATTTTTCCGATTTTG-3'		

Results

Sixty-eight (6.7%; CI: 5.3-8.5%) dogs were PCR-positive to at least one of the tested species, genera or complex of CVBD agents (Table 1). Nineteen (1.9%; CI: 1.1-2.9%) dogs were positive to *Anaplasma* spp./*Ehrlichia* spp., eight (0.8%; CI: 0.3-1.5%) to *B. burgdorferi* s.l., 31 (3.1%; CI: 2.1-4.3) to *Hepatozoon* spp. and 11(1.1%; CI: 0.5-1.9) to *L. infantum* (Table 3). *Wolbachia* spp. DNA (amplified with the same primers used to detect *Anaplasma* spp./*Ehrlichia* spp.) was detected in four dogs, while DNA of *Bartonella* spp. or *Babesia* spp. was not amplified from any dog in the study.

As shown in Table 1, the prevalence of *Anaplasma* spp/*Ehrlichia* spp. was statistically higher in domestic dogs. Positivity to these bacteria and to *Hepatozoon* spp. was higher in dogs living in the Algarve region. Statistically significant differences were also found for PCR positivity to at least one of the studied agents in domestic dogs, in dogs with access to outdoors and in dogs living in the Algarve region.

Sequencing confirmed *A. platys* in five, *E. canis* in five, *B. burgdorferis*.l. in six and *H. canis* in 18 dogs, including one animal with both *A. platys* and *H. canis* (Table 3); and revealed *Wolbachia* spp. (DDBJ accessions: LC018189 to LC018192) in four dogs.

Discussion

This is the most comprehensive study carried out in dogs from southern Portugal on the prevalence of infection

Table 3 Single and mixed PCR-positivity to species, genera and/or complex of CVBD agents in 1,010 dogs from southern Portugal

Agents	No. positive dogs (%)	DDBJ accessions
Single infections	67 (6.6)	
Anaplasma spp./*Ehrlichia* spp.	18 (1.8)	
[*Anaplasma platys*]	[4 (0.4)]	[LC018179 to LC018182]
[*Ehrlichia canis*]	[5 (0.5)]	[LC018184 to LC018188]
Borrelia burgdorferi s.l.	8 (0.8)	[LC018211 to LC018216]
Hepatozoon spp.	30 (3.0)	
[*Hepatozoon canis*]	[17 (1.7)]	[LC018193 to LC018209]
Leishmania infantum	11 (1.1)	
Co-infections	1 (0.1)	
Anaplasma spp./*Ehrlichia* spp. and *Hepatozoon* spp.	1 (0.1)	
[*A. platys* and *H. canis*]	[1 (0.1)]	[LC018183 and LC018210]
Total	68 (6.7)	

with CVBD agents as it included domestic and stray animals with and without clinical signs compatible with a vector-borne disease. DNA from these pathogens taken all together was less frequently detected in dogs (6.7%; $p < 0.001$) than in cats (29.9%; 194/649) from the same region [26]. Furthermore, only one (0.1%) dog was found co-infected (with two pathogens), whereas 29 (4.5%) cats were positive to two agents and four (0.6%) cats to three agents [26].

In this study *A. platys* has been molecularly confirmed to infect dogs from the south of the country, corroborating previous detection of this bacterium in dogs [11,23] and in *R. sanguineus* [32] from the same region. The prevalence of positivity to *Anaplasma*/*Ehrlichia* in this work (1.9%) was lower than the 4.0% obtained in Spain [33] and the 3.7-6.0% in Italy [34], which might be related with the targeted population. In fact, in the works of Tabar et al. [33] and Trotta et al. [34], all the positive dogs were sick animals with clinical signs compatible with vector-borne diseases and admitted for medical treatment, while in the present work most of the enrolled animals were apparently healthy. Interestingly, in our study most of the animals harbouring *Anaplasma*/*Ehrlichia* DNA were from Faro, overlapping the Algarve region, the southern most district of continental Portugal, which seem to follow the trend revealed by Cardoso et al. [3] that the prevalence of antibodies against *Anaplasma* spp. and *E. canis* in dogs from southern Portugal was significantly higher than in dogs from the northern and central regions of the country.

In the present work, *B. burgdorferi* s.l. DNA was amplified from 0.8% of the screened animals, providing the first molecular evidence of naturally occurring *B. burgdorferi* s.l. infection in dogs from Portugal. The exposure of dogs to these spirochetes was previously demonstrated by specific serology in the Algarve [35] and in the Alentejo and Lisbon regions [3]. Furthermore, *B. burgdorferi* s.l. genospecies, *Borrelia lusitaniae* was isolated from humans [36-38] and DNA of *B. burgdorferi* s.l. was detected in ticks [32,39] and cats from the south of the country [26]. Nevertheless, information on the clinical signs associated with *Borrelia* infections in dogs and their role as sentinels is still limited [6].

H. canis was the most prevalent pathogen detected in all the assessed dogs, with a significantly higher prevalence in animals living in the Algarve. In fact, *H. canis* has recently been identified in dogs [23], in *R. sanguineus* collected from dogs living in this region [32], and also in foxes from the south [40], showing that the protozoan is widespread in this area of the country. Although in this study only three out of the 31 infected dogs presented clinical signs, subclinical infections should not be neglected as they may progress to a severe disease and warrant treatment [41]. Concurrent infections of *H. canis* with other canine pathogens are common [21]; however, in the present work only one animal apparently healthy was co-infected with *A. platys* and the protozoan. Although this individual dog had no clinical signs of a CVBD, co-infections may potentiate disease pathogenesis, altering clinical manifestations associated with single infections [42].

The overall prevalence of *L. infantum* infection in the present study (1.1%) was much lower ($p < 0.001$) than the 34.9% obtained in 152 dogs from Lisbon [43]. The lower detection of *Leishmania* DNA might be due to the: (i) dynamics of infection over time, which may depend on the abundance and distribution of the proven vector species in conjunction with the number of infected vertebrate hosts [44], and (ii) insufficient data regarding the duration of parasitaemia in infected dogs. In fact, and taking into account a seroprevalence of 18.2% recently obtained in 170 dogs from the Algarve region [45], PCR with blood should be used to complement serological results and not only by itself to detect *Leishmania* infection, as it can lead to false negative results, especially in subclinically infected dogs [46].

PCR-positivity to one or more genera/complex of CVBD agents was found to be associated with domestic dogs, with animals living in the Algarve and with an outdoor or mixed (i.e. with outdoor access) housing. In fact, most of the domestic dogs harbouring DNA of the studied pathogens lived in rural areas from the Algarve region and used to spend most of their time exclusively outdoors, thus increasing their exposure to arthropod vectors and the agents they might transmit.

The role of domestic dogs as reservoirs of *Bartonella* spp. is less clear than for cats, and the former are probably accidental hosts. Nevertheless, they are excellent sentinels for human infections because a similar disease spectrum develops in dogs [47]. Serologic and molecular evidence of *Bartonella henselae* and *Bartonella clarridgeiae* exist for cats from the south of Portugal [26,48]. Thus, the non-detection of *Bartonella* DNA in the present study might be related with differences in immune responses, host preference of particular vectors or innate resistance in dogs to these bacteria. On the other hand, the definitive diagnosis of *Bartonella* infection is challenging due to the fastidious nature and intracellular tropism of these bacteria for erythrocytes and endothelial cells [49]. According to Perez et al. [50], enrichment culture and subculture, followed by PCR amplification, enhances molecular diagnostic sensitivity in dogs. Thus, it is possible that the PCR done directly from blood samples might have missed some positive cases; nevertheless, the prevalence of infection at the population level, if any, must be very low.

Albeit the detection of *B. canis*, *B. vogeli* and the *B. microti*-like piroplasm has already been reported in dogs from the north of Portugal [13,19,20,22] and *B. vogeli* in dogs from the south of the country [23], in the present study none of the screened animals harboured piroplasmid DNA. The non-detection of *B. canis* could somehow be

expected as its vector, *D. reticulatus*, is more abundant in the north of the country. However, the absence of *B. vogeli* and *B. microti*-like DNA is more difficult to explain, since both have already been detected in southern Portugal, the former in cats [26], dogs [23] and ticks [32], and the latter in foxes [51]. According to a recent questionnaire-based survey on the distribution of canine babesiosis in western Europe, the annual incidence of this parasitosis in southern Spain, which is geographically close to the area surveyed in this study, was estimated to be 0.0-0.7% [52]. Furthermore, a 58% prevalence of antibodies anti-*Babesia* spp. was reported among 331 dogs from kennels/shelters in southern Portugal [53]. The absence of *Babesia* spp. infection in the present study might be related with differences in the genetic background/immune system or between vector-dog interactions. Further studies are needed to better understand the epidemiological importance of these findings.

Conclusion

The identification of CVBD agents in southern Portugal, some of them with zoonotic concern, reinforces the importance to alert the veterinary community, owners and public health authorities to prevent the risk of transmission of vector-borne pathogens among dogs and to other vertebrate hosts including humans. Interestingly, the prevalence of the selected pathogens was much lower than that previously found in cats from the same region [26], probably because veterinarians and owners are much aware of them in the canine population and prophylatic measures such as insecticides and acaricides are used.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CM planned, designed and supervised the study, and wrote the manuscript; BA, CR, and MCF collected samples and clinical data, and performed DNA extraction and molecular analyses; AM, FM, JMC, MC, NN and PS collected samples and clinical data; MN performed *B. burgdorferi* s.l. nested-PCR; LuC performed data analysis and revised the manuscript; MLV and LeC reviewed the manuscript. All authors read and approved the final manuscript.

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Bacterial and protozoal agents of feline vector-borne diseases in domestic and stray cats from southern Portugal

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Abstract

Background: Feline vector-borne diseases (FVBD) have emerged in recent years, showing a wider geographic distribution and increased global prevalence. In addition to their veterinary importance, domestic cats play a central role in the transmission cycles of some FVBD agents by acting as reservoirs and sentinels, a circumstance that requires a One Health approach. The aim of the present work was to molecularly detect feline vector-borne bacteria and protozoa with veterinary and zoonotic importance, and to assess associated risk factors in cats from southern Portugal.

Methods: Six hundred and forty-nine cats (320 domestic and 329 stray), from veterinary medical centres and animal shelters in southern Portugal, were studied. *Anaplasma* spp./*Ehrlichia* spp., *Babesia* spp., *Bartonella* spp., *Borrelia burgdorferi* sensu lato, *Hepatozoon* spp. and *Leishmania* spp. infections were evaluated by polymerase chain reaction (PCR) in blood samples.

Results: One hundred and ninety-four (29.9%) cats were PCR-positive to at least one of the tested genera or complex of FVBD agents. Sixty-four (9.9%) cats were positive to *Leishmania* spp., 56 (8.6%) to *Hepatozoon* spp., 43 (6.6%) to *Babesia* spp., 35 (5.4%) to *Anaplasma* spp./*Ehrlichia* spp., 19 (2.9%) to *Bartonella* spp. and 14 (2.2%) to *B. burgdorferi* s.l. Thirty-three (5.1%) cats were positive to two (n = 29) or three (n = 4) genera/complex. *Babesia vogeli*, *Bartonella clarridgeiae*, *Bartonella henselae*, *Ehrlichia canis*, *Hepatozoon felis* and *Leishmania infantum* were identified by DNA sequencing.

Conclusions: The occurrence of FVBD agents in southern Portugal, some of them with zoonotic character, emphasizes the need to alert the veterinary community, owners and public health authorities for the risk of infection. Control measures should be implemented to prevent the infection of cats, other vertebrate hosts and people.

Keywords: Cats, Feline vector-borne diseases, Bacteria, Protozoa, Portugal

Background

Vector-borne diseases comprise a group of globally distributed and rapidly spreading illnesses that are caused by a range of pathogens transmitted by arthropods, including ticks, fleas, mosquitoes and phlebotomine sand flies [1-3]. In addition to their veterinary importance, cats and dogs play a central role in the transmission cycles of some agents of vector borne diseases (e.g. anaplasmosis, bartonellosis, borreliosis and leishmaniosis) by acting as reservoirs, amplifying hosts or sentinels, with such circumstances requiring a One Health approach [4,5].

Feline vector borne diseases (FVBD) have emerged in recent years, showing a wider geographic distribution and increased global prevalence. Environmental, demographic and human behavioral factors (e.g. travelling with pets, changes in social and leisure activities), together with the direct impact of climate changes on the abundance,

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geographical distribution and vectorial capacity of vector arthropods have contributed to the changing epidemiology of these arthropod-borne diseases [2,6].

The detection of FVBD agents can be challenging as some of them occur in healthy cats, and the clinical signs, whenever present, are normally unspecific of those diseases. PCR-based methods applied to vector-borne pathogens are very effective to detect and characterize infecting organisms, for monitoring cure after chemotherapy and to evaluate the role that subclinically-infected cats can play in the transmission of infections [7].

A recent polymerase chain reaction (PCR) study reported positivity to *Anaplasma/Ehrlichia*, *Babesia*, *Hepatozoon*, *Leishmania* and *Rickettsia* in client-owned cats from the north and centre regions of Portugal [8]. Molecular and serological studies on *Leishmania infantum* [9,10], *Anaplasma phagocytophilum*, *Bartonella* spp. and *Rickettsia conorii* [11] have been performed in domestic and stray cats from southern Portugal. Nevertheless, information about FVBD agents circulating countrywide is still limited, especially in the south, and therefore the aim of the present study was to assess the presence of bacteria and protozoa with veterinary and zoonotic importance in cats from southern of Portugal, and to assess positivity-associated risk factors.

Methods

Cats and samples

From January 2012 to August 2013, a total of 649 cats (320 domestic and 329 stray), from veterinary medical centres and animal shelters in southern Portugal, were studied. Cats were from the districts of Lisbon (n = 282) and Setúbal (n = 104), in the region of Lisbon, and from the district of Faro (n = 263), which overlaps the region of the Algarve. In the Lisbon region most of the domestic cats lived in apartments or in semi-detached houses, while cats from the Algarve region lived in rural areas and used to spend most of their time exclusively outdoors.

Out of the stray cats, 294 were collected to be neutered for birth-rate control or to be housed in a shelter for adoption, and 35 were captured and euthanized in the scope of official animal control programs. Domestic cats were randomly included after obtaining the owners informed consent. In the case of stray cats, written consent for enrolment was also obtained from the person in charge of each shelter.

Whole blood samples were collected by cephalic or jugular venipuncture and spotted onto filter paper (Whatman no. 3) for DNA extraction. Samples were dried at room temperature and kept at 4°C until tested. Whenever available, data on gender, breed, living conditions, age, use of acaricides/insecticides, clinical status (presence or absence of clinical signs compatible with a FVBD), and serological status regarding feline immunodeficiency virus (FIV) and feline leukaemia virus (FeLV) infections were registered for each cat (Table 1). Clinical signs compatible with FVBD comprised anorexia, muscular atrophy, dermatological manifestations, exercise intolerance, fever, gastrointestinal alterations, lameness, lymphadenopathy, muscular lethargy, ocular manifestations, pale mucous membranes or weight loss.

This study was ethically approved by the boards of the Institute of Hygiene and Tropical Medicine (IHMT-UNL) and of the Faculty of Veterinary Medicine (ULHT) as complying with the Portuguese legislation for the protection of animals (Law 92/1995).

PCR amplification and sequencing

A commercial kit (Kit Citogene®, Citomed) was used to extract DNA from blood on filter paper. Four discs of filter paper (4 mm in diameter each) were incubated with lysis buffer (150 μl) and 1.5 μl of proteinase K (20 mg/ml). Further DNA extraction followed the kit manufacturer's instructions.

Positivity to *Anaplasma* spp./*Ehrlichia* spp., *Babesia* spp., *Bartonella* spp., *B. burgdorferi* s.l., *Hepatozoon* spp. and *Leishmania* spp. DNA in blood samples was tested by PCR according to previously described protocols (Table 2). PCR amplifications were performed in a 25 μl final volume containing 2 mM $MgCl_2$, 1 unit of Taq DNA polymerase (GoTaq DNA Polymerase®, Promega), 10 pmol of each primer (15 pmol in the case of *L. infantum*), 0.2 μM each of dATP, dTTP, dCTP and dGTP (Dntps set®, Bionline, Citomed), and 3 μl of DNA template (5 to 200 ng). In all amplifications a positive control containing genomic target DNA and a negative control without DNA were included. PCR products were visualized under UV illumination after electrophoresis migration on a 1.5% gel agarose stained with 0.2 mg/ml ethidium bromide, using a 100 bp DNA ladder as a marker.

Twenty per cent of the PCR products (30% in the case of *Bartonella* spp.) were purified with a High Pure PCR Product Purification Kit (Roche® Mannheim) according to the manufacturer's instructions and directly sequenced (one direction) (Stabvida®), using the same primers as those used for the DNA amplification. Species identity of the obtained sequences was determined according to the closest BLAST match (identity ≥97%) to a GenBank accession and deposited in DNA Data Bank of Japan (DDBJ) (http://www.DDBJ.nig.ac.jp).

Statistical analysis

Percentages of positivity to FVBD agents relative to the independent variables and categories (Table 1) were compared by the Chi-square or Fisher's exact tests. A p value <0.05 was considered as statistically significant. Analyses were performed with SPSS® 21 software for Windows.

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Table 1 Comparison of prevalence of FVBD pathogens in different groups of cats from southern Portugal

Variable/ category	Nº of tested cats (%)	Nº of positive cats (%)						
		Anaplasma/Ehrlichia	*Babesia*	*Bartonella*	*B. burgdorferi* s.l.	*Hepatozoon*	*Leishmania*	≥ 1 positive PCR
Gender	649							
Female	372 (57.3)	18 (4.8)	31 (8.3)	11 (3.0)	9 (2.4)	32 (8.6)	44 (11.8)	122 (32.8)
Male	277 (42.7)	17 (6.1)	12 (4.3)	8 (2.9)	5 (1.8)	24 (8.7)	20 (7.2)	72 (26.0)
Breed	484							
DSH	432 (89.3)	22 (5.1)	22 (5.1)*	10 (2.3)	4 (0.9)	31 (7.2)	33 (7.6)	109 (25.2)
Other breed	52 (10.7)	1 (1.9)	10 (19.2)*	2 (3.8)	1 (1.9)	3 (5.8)	7 (13.5)	19 (36.5)
Lifestyle	649							
Domestic	320 (49.3)	9 (2.8)*	28 (8.8)*	5 (1.6)	3 (0.9)	24 (7.5)	33 (10.3)	91 (28.4)
Stray	329 (50.7)	26 (7.9)*	15 (4.6)*	14 (4.3)	11 (3.3)	32 (9.7)	31 (9.4)	103 (31.3)
Age (months)	462							
[3-11]	129 (27.9)	5 (3.9)	13 (10.1)	4 (3.1)	1 (0.8)	3 (2.3)*	6 (4.7)[a]	27 (20.9)
[12-59]	216 (46.8)	10 (4.6)	13 (6.0)	6 (2.8)	0 (0.0)	17 (7.9)	14 (6.5)[b]	54 (25.0)
[60-228]	117 (25.3)	5 (4.3)	5 (4.3)	0 (0.0)	2 (1.7)	11 (9.4)*	16 (13.7)[a,b]	35 (29.9)
Acaricides-insecticides	568							
Yes	204 (35.9)	5 (2.5)	4 (2.0)*	3 (1.5)	3 (1.5)	11 (5.4)*	27 (13.2)*	51 (25.0)*
No	364 (64.1)	22 (6.0)	37 (10.2)*	16 (4.4)	10 (2.7)	42 (11.5)*	28 (7.7)*	126 (34.6)*
Region	649							
Lisbon	386 (59.5)	18 (4.7)	9 (2.3)*	8 (2.1)	3 (0.8)*	25 (6.5)*	31 (8.0)	85 (22.0)*
Algarve	263 (40.5)	17 (6.5)	34 (12.9)*	11 (4.2)	11 (4.2)*	31 (11.8)*	33 (12.5)	109 (41.4)*
Habitat	649							
Urban	282 (43.5)	18 (6.4)	8 (2.8)*	4 (1.4)	0 (0.0)	14 (5.0)*	9 (3.2)*	47 (16.7)*
Rural	367 (56.5)	17 (4.6)	35 (9.5)*	15 (4.1)	14 (3.8)	42 (11.4)*	55 (15.0)*	147 (40.1)*
Housing	589							
Totally indoors	124 (21.1)	3 (2.4)	6 (4.8)	1 (0.8)	0 (0.0)	3 (2.4)*	13 (10.5)	24 (19.4)*
Access to outdoors	465 (78.9)	28 (6.0)	36 (7.7)	17 (3.7)	8 (1.7)	51 (11.0)*	39 (8.4)	151 (32.5)*
FeLV	242							
Negative	231 (95.5)	12 (5.2)	15 (6.5)	6 (2.6)	6 (2.6)	13 (5.6)	29 (12.6)	70 (30.3)
Positive	11 (4.5)	1 (9.1)	1 (9.1)	0 (0.0)	1 (9.1)	1 (9.1)	0 (0.0)	3 (27.3)
FIV	247							
Negative	226 (91.5)	11 (4.9)	16 (7.1)	6 (2.7)	6 (2.7)	14 (6.2)	28 (12.4)	69 (30.5)
Positive	21 (8.5)	3 (14.3)	1 (4.8)	0 (0.0)	1 (4.8)	1 (4.8)	1 (4.8)	6 (28.6)
Clinical status	222							
Non-suspect	197 (88.7)	8 (4.1)	26 (13.2)	1 (0.5)	0 (0.0)	12 (6.1)	8 (4.1)	49 (24.9)
Suspect	25 (11.3)	3 (12.0)	1 (4.0)	0 (0.0)	0 (0.0)	1 (4.0)	3 (12.0)	6 (24.0)
Total	649 (100)	35 (5.4)	43 (6.6)	19 (2.9)	14 (2.2)	56 (8.6)	64 (9.9)	194 (29.9)

*,a,b Statistically significant difference ($p < 0.05$); DSH: domestic short-haired; FeLV: feline leukaemia virus; FIV: feline immunodeficiency virus.

Results

One hundred and ninety-four (29.9%) cats were PCR-positive to at least one of the tested genera or complex of FVBD agents (Table 2). Sixty-four (9.9%) cats were positive to *Leishmania* spp., 56 (8.6%) to *Hepatozoon* spp., 43 (6.6%) to *Babesia* spp., 35 (5.4%) to *Anaplasma* spp./*Ehrlichia* spp., 19 (2.9%) to *Bartonella* spp. and 14 (2.2%) to *B. burgdorferi* s.l. Thirty-three (5.1%) cats were positive to two (n = 29) or three (n = 4) genera/complex (Table 3).

As shown in Table 1, the non-use of acaricides/insecticides and living in rural areas were associated with *Babesia* spp. and *Hepatozoon* spp.. Furthermore, the prevalence

Table 2 Primer sets for PCR amplification of FVBD agents

Pathogen	Gene	Primers	Product size (bp)	PCR conditions	Reference
Anaplasma spp./*Ehrlichia* spp.	16S rRNA	EHR16SD: 5′-GGT ACC YAC AGA AGA AGTCC-3′	345	95°C, 5 min; 35 cycles [94°C 30 sec, 55°C 30 sec, 72°C 90 sec]; 72°C, 5 min	[12]
		EHR16SR: 5′-TAG CAC TCA TCG TTT ACAGC-3′			
Babesia spp.	18S rRNA	PIRO-A: 5′-AAT ACC CAA TCC TGA CACAGG G-3′	400	95°C, 5 min; 35 cycles [94°C 30 sec, 55°C 30 sec, 72°C 90 sec]; 72°C, 5 min	[13]
		PIRO-B: 5′-TTA AAT ACG AAT GCC CCCAAC-3′			
Bartonella spp.	16S-23S rRNA	325 s: 5′-CTTCAGATGATGATCCCAAGCCTTCTGGCG-3′	500-800	95°C, 5 min; 55 cycles [95°C 15 sec, 66°C 15 sec, 72°C 15 sec]; 72°C, 1 min	[14]
		1100as: 5′-GAACCGACGACCCCCTGCTTGCAAAGCA-3′			
B. burgdorferi s.l.	5S-23S rRNA	Outer primers:	380		[15]
		23SN1: 5′-ACCATAGACTCTTATTACTTTGAC-3′		94.5°C, 1 min; 25 cycles [94°C 30 sec, 52°C 30 sec, 72°C 1 min]; 72°C, 5 min	
		23SC1: 5′-TAAGCTGACTAATACTAATTACCC-3′			
		Inner primers:	225		
		23SN2: 5′-ACCATAGACTCTTATTACTTTGACCA-3′		94.5°C, 1 min; 40 cycles [94°C 30 sec, 55°C 30 sec, 72°C 1 min]; 72°C, 5 min	
		5SCB: 5′-biotin-GAGAGTAGGTTATTGCCAGGG-3′			
Hepatozoon spp.	18S rRNA	HEP-F: 5′-ATA CAT GAG CAA AAT CTC AAC-3′	626-666	95 °C, 5 min; 34 cycles [95 °C 20 sec, 55 °C, 30 sec, 72 °C, 90 sec]; 72 °C, 5 min	[16]
		HEP-R: 5′-CTT ATT ATT CCA TGC TGC AG-3′			
Leishmania spp.	Small subunit rRNA	Outer primers:	603		[17]
		R221: 5′-GGTTCCTTTCCTGATTTACG-3′		94°C, 5 min; 35 cycles [94°C 30 sec, 60°C 30 sec, 72°C 30 sec]; 72°C, 10 min	
		R332: 5′-GGCCGGTAAAGGCCGAATAG-3′			
		Inner primers:	358		[18]
		R223: 5′-TCCCATCGCAACCTCGGTT-3′		94°C, 5 min; 35 cycles [94°C 30 sec, 65°C 30 sec, 72°C 30 sec]; 72°C, 10 min	
		R333: 5′-AAAGCGGGCGCGGTGCTG-3′			

Table 3 Single and mixed PCR-positivity to genera (*Anaplasma*/*Ehrlichia*, *Babesia*, *Bartonella*, *Hepatozoon* and *Leishmania*) and/or complex (*B. burgdorferi* s.l.) of FVBD agents in 649 cats from southern Portugal

Agent(s)	No. of cats (%)
Single positivity	161 (24.8)
Anaplasma spp./*Ehrlichia* spp.	24 (3.7)
Babesia spp.	28 (4.3)
Bartonella spp.	10 (1.5)
B. burgdorferi s.l.	8 (1.2)
Hepatozoon spp.	37 (5.7)
Leishmania spp.	54 (8.3)
Mixed positivity	33 (5.1)
Anaplasma spp./*Ehrlichia* spp. + *Bartonella* spp.	2 (0.3)
Anaplasma spp./*Ehrlichia* spp. + *B. burgdorferi* s.l.	1 (0.2)
Anaplasma spp./*Ehrlichia* spp. + *Hepatozoon* spp.	2 (0.3)
Anaplasma spp./*Ehrlichia* spp. + *Leishmania* spp.	2 (0.3)
Babesia spp. + *Bartonella* spp.	3 (0.5)
Babesia spp. + *Hepatozoon* spp.	8 (1.2)
Babesia spp. + *Leishmania* spp.	2 (0.3)
Bartonella spp. + *B. burgdorferi* s.l.	1 (0.2)
Bartonella spp. + *Hepatozoon* spp.	2 (0.3)
B. burgdorferi s.l. + *Hepatozoon* spp.	1 (0.2)
B. burgdorferi s.l. + *Leishmania* spp.	3 (0.5)
Hepatozoon spp. + *Leishmania* spp.	2 (0.3)
Anaplasma spp./*Ehrlichia* spp. + *Babesia* spp. + *Hepatozoon* spp.	2 (0.3)
Anaplasma spp./*Ehrlichia* spp. + *Bartonella* spp. + *Hepatozoon* spp.	1 (0.2)
Anaplasma spp./*Ehrlichia* spp. + *Hepatozoon* spp. + *Leishmania* spp.	1 (0.2)
Total	194 (29.9)

of *Babesia* spp. and *Anaplasma* spp/*Ehrlichia* spp. was statistically higher in domestic and stray cats, respectively. Cats from exotic breeds (including their crosses) had higher positivity to *Babesia* spp. than domestic short-haired cats. Prevalence of *Hepatozoon* spp. was higher in cats with access to outdoors and in cats older than 60 months (5 years). *Leishmania* spp. was more prevalent in cats aged 12-59 months and in cats aged 60-228 months than in cats younger than 12 months, in cats living in rural habitats and in those protected against ectoparasites. Positivity to *Babesia* spp., to *Hepatozoon* spp. and to *Leishmania* spp. was higher in cats living in the Algarve region. Statistically significant differences were also found for PCR positivity to at least one of the studied agents in cats living in the Algarve region, in cats from rural areas, in cats with access to outdoors and in cats without protection against ectoparasites.

Sequencing confirmed *Hepatozoon felis* in 13 cats [DDBJ accession numbers: AB872992 to AB872995; AB896686 to AB896694], *Babesia vogeli* in eight [DDBJ accession numbers: AB896788 to AB896795], *Leishmania infantum* in five cats [DDBJ accession numbers: AB896681 to AB896685], *Bartonella clarridgeiae* in four [DDBJ accession numbers: AB896695 to AB896698], *Bartonella henselae* in two [DDBJ accession numbers: AB872991 and AB896699] and *Ehrlichia canis* in one cat [DDBJ accession number: AB896787]. Although sequencing results were not obtained for all the products of PCR positive reactions, mainly due to small quantities of amplified DNA, all the obtained sequences revealed an agent species consistent with the PCR result.

Discussion

The present study represents the first survey on FVBD agents performed in cats from southern Portugal. The overall prevalence of *Leishmania* spp. infection in the present study (9.9%) was higher than the one obtained in domestic cats from the north and centre of the country (0.3%) [8], but lower than the prevalence obtained in domestic (20.3%) and stray (30.4%) cats from Lisbon [9,10], suggesting that the rate of *Leishmania* infection might be dynamic over time, depending on the abundance and distribution of proven vector species in conjunction with the number of infected vertebrate hosts. The significant differences of *Leishmania* spp. prevalence between juvenile and adult or old cats corroborated the results obtained in cats from the north of the country [19] and match the situation previously found in a national serosurvey of *Leishmania* canine infection [20]. Seropositivity to *L. infantum* was significantly higher in dogs and cats older than 2 years of age [19,20], which may probably be explained by a cumulative exposure of older animals to the protozoan parasite. The increased contact with the vectors might also be the reason for a significantly higher prevalence of *Leishmania* infection in the surveyed cats living in a rural environment [20].

L. infantum has been reported in cats co-infected with immunosuppressive viruses [21]. However, in this study only one cat was co-infected with FIV, corroborating other studies where no association was observed between the presence of *Leishmania* and of FeLV or FIV infections [10].

The use of topical insecticides on dogs has been shown to be effective in reducing the incidence of canine and human visceral infections. However, in the present work cats treated with acaricides/insecticides presented a higher prevalence of positivity to *Leishmania*. Although the compliance of ectoparasiticide application was not evaluated, this result is not entirely surprising because, even if owners had regularly administered insecticides/acaricides, the only repellents effective against sand flies, the pyrethroids, are toxic to cats. A trend to consider cats as a domestic reservoir of *L. infantum* exists, as infection in

domestic and stray cats has been increasingly reported in endemic areas [22]. The potential role of cats in zoonotic leishmaniosis, together with the inexistence of suitable repellents that can be used on cats against sand flies, is a critical issue that should be addressed to prevent feline *Leishmania* infection [22].

The detection of *Hepatozoon* spp. and *H. felis* in cats from southern Portugal reported in the present study, together with the sequenced genetic variants of *H. felis* from cats living in the north and centre [8] suggest that the protozoan is widespread throughout the country. The vectors and route of infection of *H. felis* remain unknown [23], although it was recently amplified from *Rhipicephalus sanguineus* collected from cats and dogs living in the centre and south of Portugal [24].

Sporadic cases of *Babesia canis* and the *Babesia microti*-like piroplasm (syn. *Theileria annae*) infections were reported in three immunocompromised domestic cats from Portugal [25,26]. The overall prevalence of *Babesia* infection (6.9%) obtained in the present study was similar to the 9.1% obtained by Vilhena *et al.* [8], corroborating that piroplasmid infections in cats are frequent and that *B. vogeli* is probably the most common species circulating in felines in Portugal. Cats from the Algarve region, those living in rural habitats or not treated with acaricides/insecticides had a significantly higher prevalence of *Babesia* spp. infection in comparison with cats living in the Lisbon region or in urban areas or chemically protected against ectoparasites, probably due to a higher exposition of the former to the vectors. Differences in the genetic/immune background could be the reason why exotic breeds (including their crosses) presented a higher predisposition of positivity to piroplasms. As the clinical importance of infection with most *Babesia* species in cats remains unknown, as well as the vectors responsible for their transmission [26], further studies are needed to understand the epidemiological relevance of piroplasm infection in the feline population.

Several pathogens belonging to the genera *Anaplasma* and *Ehrlichia* are shared by man and companion animals [1], and there is serological and molecular evidence that cats can be infected with species of these intracellular bacteria [7,21,27]. In fact, antibodies to *A. phagocytophilum* and *E. canis* and DNA of *Anaplasma*/*Ehrlichia* were previously detected in cats from Portugal [8,11,28]. Nonetheless, and to the best of our knowledge, this is the first time in the country that *E. canis* has been molecularly confirmed to infect cats.

The prevalence of positivity to *Anaplasma*/*Ehrlichia* in this work (5.4%) was higher than the 1.0% obtained in Spain [27] and than the 0.6% obtained in cats from the centre and north of Portugal [8]. These differences can be related to the targeted population, as only client-owned cats were evaluated in the two above-mentioned studies. In fact, the prevalence of *Anaplasma*/*Ehrlichia* infection was significantly higher in stray cats in the present study. On the other hand, the seroprevalence of *Ehrlichia* infection in stray cats from the Madrid region was lower than in owned cats [21], thus highlighting that other factors favoring vector-host interactions, such as vector density and geographic distribution, and host immunological status, might play a role in the prevalence of feline ehrlichiosis. Interestingly, our results in combination with those from Vilhena *et al.* [8] seem to follow the trend of significantly higher prevalences of antibodies to *Anaplasma* spp. and *E. canis* in dogs from southern Portugal than in dogs from the northern and central regions of the country [3].

Subclinical infection with *B. clarridgeiae* or *B. henselae*, agents of the cat scratch disease, is frequently reported in cats, which are therefore regarded as a major reservoir for human infection [27,29,30]. Recognised risk factors for bacteraemia in cats are young age (<12 months), infestation with fleas, an outdoor lifestyle and a multicat environment [11,29,30]. Data obtained in the present study corroborates these findings, as most of the cats PCR-positive to *Bartonella* spp. were stray cats and/or with access to outdoors and were not protected against ectoparasites. We report the first molecular evidence of *B. clarridgeiae* infection in cats from Portugal. So far, *B. clarridgeiae* had only been previously detected in *Ctenocephalides felis* fleas from Lisbon and Évora districts [11]. The prevalence of *Bartonella* spp. obtained in the present study (2.9%) was higher than the prevalence (0.3%) described in cats from Madrid, Spain [21], but considerably lower than the one previously reported in cats from Portugal (67.6%) [11]. Prevalence of *B. henselae* (0.3%) was also much lower than the ones previously obtained in Portugal (8.1%) [11] and in Barcelona, Spain (17.5%) [27], while prevalence of *B. clarridgiae* infection (0.6%) was similar to a study conducted in Barcelona (1.0%) [7]. Differences in prevalence could be due to climatic and environmental differences among study areas, which result in more frequent flea infestation or a higher level of *Bartonella* spp. infection among both cats and fleas [21,27].

Borreliosis (or Lyme disease) due to the spirochete *B. burgdorferi* continues to receive intense attention in the milieu of companion animals. Domestic cats are exposed to *B. burgdorferi*, with reported seroprevalence rates of 47-71% in cats from endemic areas of the northeastern USA [31]. Regarding Europe, and to the best of our knowledge, only Shaw *et al.* [32] reported *B. burgdorferi* s.l. infection by PCR, in two clinically suspected cats from the United Kingdom. In the present work, *B. burgdorferi* s.l. DNA was amplified from 2.2% of the screened cats, providing the first molecular evidence of naturally occurring *B. burgdorferi* s. l. infection in cats from Portugal. Nevertheless,

the situation of *Borrelia* infection transmission and clinical signs in cats remains a subject for further investigation in Portugal.

The contact with arthropod-borne pathogens varies with the season and depends on the activity and abundance of competent vectors. For instance, feline seropositivities to *A. phagocytophilum* and *E. canis* antigens were shown to be higher during autumn, and in May and November, respectively [27]. As most of the blood samples analysed in the present work were collected from October to May, the effect of the different seasons in the prevalence of infection by the different pathogens was not evaluated. A rural habitat, an outdoor housing or access to outdoors, and the non-use of ectoparasiticides were found to be associated with PCR-positivity to one or more genera/complex of FVBD agents, which is related to a higher exposure of cats to arthropod vectors and the agents they might transmit. As documented for dogs, certain organisms (e.g. *B. vogeli, E. canis, H. canis* and *L. infantum*) might be associated with long-term subclinical infections [1] and, in spite of remaining apparently healthy for months or even years, infected cats might serve as reservoirs to other hosts including humans.

Co-infections with different canine vector-borne pathogens are frequent in dogs living in geographic areas where the presence of competent vectors for the different pathogens overlap [1]. In previous entomological surveys made in the south of Portugal, *L. infantum* was amplified in phlebotomine sand flies [33], *Bartonella* was molecularly detected in *C. felis* [11], while *R. sanguineus* specimens were found to harbour *Anaplasma/Ehrlichia*, *Babesia*, *Borrelia* or *Hepatozoon* DNA [24]. Thus, the detection in the present study of 33 cats co-infected with two or three agents/complex of FVBD is not surprising. Nevertheless, it is important to keep in mind that the occurrence of different combinations of vector-borne pathogens, with a possible dysregulation of the immune system, may lead to a severe and non-characteristic clinical outcome which will further complicate the diagnosis, treatment and prognosis.

Conclusion

In conclusion, the wide spectrum of FVBD agents identified in southern Portugal, some of them of zoonotic concern, reinforces the importance to alert the veterinary community, owners and public health authorities for the risk of transmission of vector-borne pathogens. Therefore, effective prophylactic measures, such as the use of ectoparasiticides against arthropods, and education and awareness, must be put in place, in order to prevent infection and avoid the dissemination of these pathogens among cats and to other vertebrate hosts including human beings.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
CM planned, designed and supervised the study, and wrote the manuscript; CR, FB and PP collected samples and clinical data, and performed DNA extraction and molecular analyses; AM and MC collected samples and clinical data; MN performed *B. burgdorferi* s.l. nested-PCR; LuC performed data analysis and revised the manuscript; MLV and LeC reviewed the manuscript. All authors read and approved the final manuscript.

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Chapter 5

Development of new molecular tools for the identification of *Borrelia burgdorferi* s.l. genospecies to improve Lyme disease diagnosis

5. Development of new molecular tools for the identification of *Borrelia burgdorferi* s.l. genospecies to improve Lyme disease diagnosis

Major advances in laboratory testing for Lyme disease have occurred in recent years, although, progress in this area is still a necessity. Improvements of several aspects of the current recommended testing algorithm are needed, and also in the direct methods for detection of *B. burgdorferi* spirochetes. Techniques more accurate, sensitive, and rapid, preferably point-of-care tests, for Lyme disease diagnosis must be developed. Therefore, the current chapter describes two novel molecular tools for the detection and identification of *B. burgdorferi* s.l. bacteria, to help in a better diagnosis of Lyme disease.

This chapter is based on the research papers:

Nunes M, Parreira R., Carreira T, Inácio J, Vieira ML. 2016. Development and evaluation of a two-step multiplex *TaqMan* real-time PCR assay for detection of *Borrelia burgdorferi* s.l. genospecies.
(*in submission)*

Nunes M, Nascimento M, Carreira T, Vieira ML. 2016. Development of a Loop Mediated Isothermal Amplification (LAMP) method for the detection of *Borrelia burgdorferi* s.l. genospecies DNA in tick samples.
(*in preparation*)

5.1 – Development and evaluation of a two-step multiplex *TaqMan* real-time PCR assay for detection of *Borrelia burgdorferi* s.l. genospecies

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Abstract

Nowadays at least four clinically important *B. burgdorferi* sensu lato (s.l.) genospecies, *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s. and *B. lusitaniae* can be seen in Portugal, each one of them with different tropisms that results in diverse clinical manifestations. The standard diagnostic procedure is normally simple, if a positive history of tick exposure or a typical erythema *migrans* appears. However, during the "window-period" phase, where no antibodies are yet produced, the diagnosis is quite difficult, and more reliable and precise laboratory methods, like molecular tests, are necessary.

Several molecular methods have been developed and applied to *Borrelia* genospecies identification, however, most of them do not allow the detection of several genospecies simultaneously. The aim of this study was to implement and evaluate a combined multiplex *TaqMan* real-time PCR assay to infer the presence of *B. burgdorferi* s.l. genospecies in clinical and ixodid samples. The assay consists in two steps: (i) a first duplex real-time PCR targeting both the *flaB* gene of *B. burgdorferi* s.l., and an internal control namely the 18S rDNA for tick samples or the mammal *β-actin* gene for clinical samples; and (ii) a second tetraplex real-time PCR targeting the *flaB* gene of *B. afzelii*, *B. garinii*, *B. lusitaniae* and *B. burgdorferi* s.s..

The first duplex step reveled a high specificity and a sensitivity as low as 10 genome equivalents (GE) for *B. burgdorferi* s.l. genospecies and also for experimentally inoculated human serum samples. The second tetraplex step also revealed a high specificity, and a sensitivity of 10^2 GE for the four genospecies. This same result was obtained in experimentally inoculated sera.

The possibility to detect and identify four of the most prevalent *B. burgdorferi* s.l. genospecies in Europe in a single run is very important, since it allows to save time and reduce costs. This technology can be applied to both environmental and clinical samples, in the latter case being particularly advantageous in an early stage of Lyme disease.

Introduction

Lyme disease (LD) is now recognized as the most common vector-borne disease in both Europe and North America, with 85 000 cases estimated annually in Europe and 300 000 cases estimated annually in the USA (Lindgren & Jaenson, 2006; Hinckley et al., 2014). The disease is also present in Asia, Africa and Oceania, showing a great regional variation (Stanek & Strle, 2003; Stanek & Reiter, 2011). It is caused by spirochetes of *Borrelia burgdorferi* sensu lato (*B. burgdorferi* s.l.) complex through the bite of the hard-tick *Ixodes ricinus*. Nearly all human infections are caused by three *B. burgdorferi* s.l. genospecies: *B. garinii*, *B. afzelii*, and *B. burgdorferi* sensu stricto (s.s.) (Baranton et al., 1992). All three species cause LD in Europe, whereas only *B. burgdorferi* s.s., causes LD in the USA (Stanek et al., 2012).

The clinical manifestations of LD are broad and associated with distinct tissue or systems tropisms (van Dam et al., 1993; Balmelli & Piffaretti, 1995). Early localized infection typically results in erythema migrans (EM) rash, after which spirochetes can disseminate to the nervous system, joints, and other organs. *B burgdorferi* s.s. is normally associated with arthritis, *B. garinii* with neurological effects (musculoskeletal and nervous systems), and *B. afzelii* with skin complications, like acrodermatitis chronica atrophicans (ACA) (van Dam et al., 1993). Nevertheless, LD may also be latent, without unequivocal clinical symptoms or may present unspecific symptoms such as headache, myalgia, arthralgias or fever (Smith et al., 2002; Steere et al., 2003).

Currently the standard diagnosis of LD is quite simple, if a positive history of tick exposure or typical EM appears, being the laboratory testing unnecessary. When a laboratory confirmation of the diagnosis is needed, several methods are available being classified as direct for the detection of *B. burgdorferi* s.l. spirochetes, or indirectly for the detection of antibodies anti-*B. burgdorferi* s.l. class IgM and IgG. The direct methods include the dark-field microscopic examination, conventional polymerase chain reaction (PCR) and *in vitro* culture (Gaumond et al., 2006); and the indirect methods include ELISA, EIA and line-blots tests (Robertson et al., 2000; Steere et al., 2008; Hinterseher et al., 2012; Liu et al., 2013). However, the serological tests available are frequently unsuitable or insufficient as they can present high cross-reactivity with antigens of other pathogens, being unable to distinguish between primary or recurrent infections, and useless during the “Window period” (Marques, 2015).

Consequently, attempts to introduce new diagnostic tests for the detection and monitoring of *B. burgdorferi* s.l. infections are being made all over the world, where PCR assays proved to be a useful tool for this purpose, allowing to detect the spirochete without resorting to cultivation procedures. Several PCR methods have been developed targeting a wide range of *B. burgdorferi* s.l. genes, like the chromosomal genes recombinase A (*rec*A), the flagellin gene (*fla*B), the 16S rDNA gene, the rrs-rrlA intergenic spacer (16S-23S IGS), and the the plasmid-carried outer protein (*osp*) genes *osp*A and *osp*C (Schmidt, 1997; Lebech, 2002). The heterogeneity among *Borrelia* species in the *rec*A and *fla*B genes is sufficient to separate different species from each other by targeting heterogeneous alleles.

In the last decades the real-time PCR has been replacing the conventional PCR, due to its rapidness, high efficiency and sensitivity. This technique uses a combination of specific primers and fluorescent probes where no post amplification detection is needed. Besides, it allows to simultaneously detect several genes from different pathogenic agents in a single run, contributing for the time and cost decreasing (Edwards & Gibbs, 1994; Wittwer et al., 2001). The aim of this study was to develop an easy-to-use *TaqMan* real-time PCR assay with high sensitivity and specificity to detect *B. burgdorferi* s.l. genospecies. The assay starts with a duplex step targeting the *flaB* gene for the detection and quantification of *B. burgdorferi* s.l. genospecies, followed by a tetraplex step targeting the same gene, for the detection and quantification of four of the main genospecies present in Portugal: *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s. and *B. lusitaniae*.

Material and Methods

Design of TaqMan probes and respective flanking primers

Several sequences from *flaB* gene were retrieved from GenBank, namely *B. burgdorferi* s.s. B31 (accession number CP009656.1), *B. garinii* SZ (accession number CP007564.1), *B. afzelii* HLJ01 (accession number CP003882.1), *B. bavariensis* PBi (accession number NC_006156.1), *B. valaisiana* VS116 (accession number AB236666.1), *B. bissettii* CA128 (accession number DQ393343), *B. lusitaniae* PoTiB1 (accession number DQ111035.1), *B. californiensis* CA446 (accession number DQ393347.1), *B. spielmanii* A14S (accession number ABKB020000003.1) and *B. sinica* CMN3 (accession number AB022138.1). The

primers and *TaqMan* probes were designed to be specific for *B. burgdorferi* s.l. complex, in a first approach, and also for *B. burgdorferi* s.s., *B. afzelii*, *B. garinii* and *B. lusitaniae* simultaneously in a second approach, by using Mega 6, Primer Express software 3.0 (Applied Biosystems) and the BLAST basic local alignment search tool (Altschul et al., 1997) (Table 1). *TaqMan* probes were labelled with fluorophore with different emission spectra (Table 1). Internal controls were also designed to detect inhibition of the PCR reactions when using DNA extracted from clinical samples or ixodids, as template. Therefore, for ixodids samples *TaqMan* probe and flaking primers were design for 18S rDNA gene, and for clinical samples a *β-actin* gene-targeted mammals-universal *TaqMan* probe and flanking degenerated primers were retrieved from Costa et al., 2013 (Table 1).

Table 1 – Sequences of primers and probes designed in this study.

Primers/Probes	Primer/Probe sequence (5'- 3')	Complementary target
F_*Bb*sl	TTAATGTTACAACYACAGTTGA	*flaB* gene for *B. burgdorferi* s.l. complex
R_*Bb*sl	GCTACAATGACAGATGAGGT	
P_*Bb*sl[1]	JOE-AAGAGCAAATTTAGGTGCTTTCCAA – BHQ1	
***F_B*spp**	CAAGATGAAGCDATTGCTGTAAA	*flaB* gene for *B. afzelli, B. garinii, B. lusitaniae, B. burgdorferi* s.s.
***R_B*spp**	CTGCTACAGCACCTTCTCA	
P_*Bafz*[2]	ROX – TTCTTGAGCACCCTCTTGAACAGG – BHQ2	
P_*Bgar*[3]	Cy5 – CTTGTTGAGCTCCTTCTTGAACAGG – BHQ2	
P_*Blus*[4]	JOE – TTGAACACCTTCTTGAGCAGGTGCA – BHQ1	
P_*Bb*ss[1]	FAM –TCCTTCCTGTTGAACACCCTCTTG – BHQ1	
F_*B*-actin	GGCTCYATYCTGGCCTC	*β-actin* gene of mammals
F_*B*-actin	GCAYTTGCGGTGSACRATG	
P_*B*-actin[1]	FAM – TACTCCTGCTTGCTGATCCACATC – BHQ1	
F_18S rRNA	AGCTAATACATGCAGTGAGC	18S rDNA gene of ixodids
R_18S rRNA	TGATCGCATGGCCACGAG	
P_18S rRNA[1]	FAM – CGGGTGCTTTTATTAGACCAAGAT – BHQ1	

*Bb*sl – *Borrelia burgdorferi* sensu lato; Bafz – *Borrelia afzelii*; Bgar – *Borrelia garinii*; Blus – *Borrelia lusitaniae*; Bbss – B*orrelia burgdorferi* sensu stricto;[1] Probe labeled with FAM (carboxyfluorescein) fluorophore and BHQ1 quencher; [2]Probe labeled with ROX (6-carboxyX-rhodamine) fluorophore and BHQ2 quencher; [3]Probe labeled with Cy5 (Cyanine) fluorophore and BHQ2 quencher; [4]Probe labeled with JOE fluorophore and BHQ1 quencher.

Two-step multiplex real-time PCR algorithm

The amplification-based identification algorithm using DNA extracted from *B. burgdorferi* s.l. reference strains as template consists in two steps: (i) a first duplex real-time PCR targeting the *flaB* gene of *B. burgdorferi* s.l. and the internal control 18S rDNA for hard-ticks samples, or *β-actin* gene for clinical samples; and (ii) a second tetraplex real-time PCR targeting the *flaB* gene of *B. afzelii*, *B. garinii*, *B. lusitaniae* and *B. burgdorferi* s.s. (Figure 1). All the amplification reactions were optimized using DNA extracted from *B. burgdorferi* s.l. cultures as template.

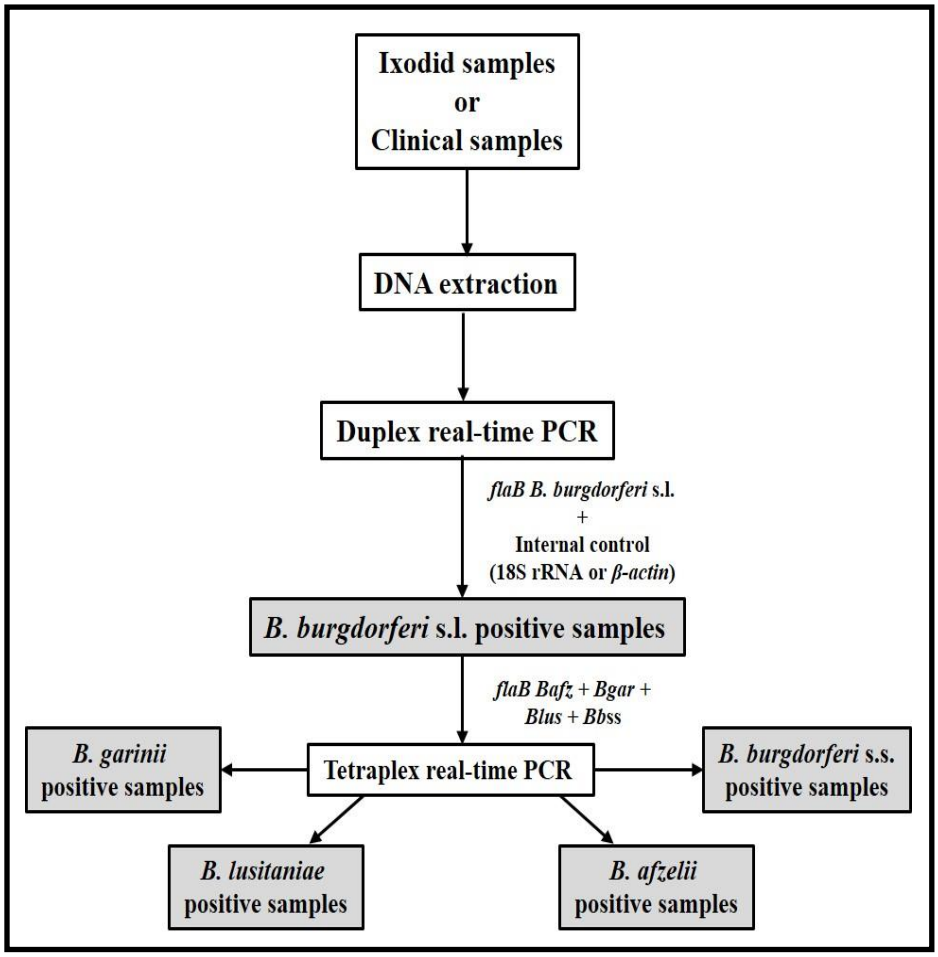

Figure 1 – Representation of the real-time PCR algorithm for identification of *B. burgdorferi* s.l. genospecies. The targeted genes are indicated above the arrows (B. – *Borrelia*; *Bafz* – *B. afzelii*; *Bgar* – *B. garinii*; *Blus* – *B. lusitaniae*; *Bbss* – *B. burgdorferi* s.s.).

Duplex real-time PCR reactions were carried out in a total volume of 20 µl containing 1× SensiFAST™ (Bioline), 0.3 µM of each primer (F_*Bb*sl, R_*Bb*sl; F_18S rRNA, r_18S rRNA or F_*β-actin*, R_ *β-actin*), 0.25 µM of each *TaqMan* probe (P_*Bb*sl; P_18S rRNA or P_ *β-actin*), DNase free water (Bioline) and 2 µl of the extracted DNA template. The thermal cycling conditions were: 1 cycle at 95 °C for 1 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 45 s. The tetraplex real-time PCR reactions used 1× SensiFAST™ (Bioline), 0.3 µM of F_*B*spp, R_*B*spp primers, 0.25 µM of P_*Bafz*, P_*Bgar*, P_*Bb*ss and 0.15 µM of P_*Blus TaqMan* probes, DNase free water (Bioline), and 2 µl of the extracted DNA template, in a total volume of 20 µl. The thermal cycling conditions were: 1 cycle at 95 °C for 5 min, followed by 45 cycles at 95 °C for 10 s and 60 °C for 30 s. All positive samples were retested for confirmation. Non-template negative controls (with PCR grade water) were included in each run to rule out the possibility of cross-contamination. Thermal cycling, fluorescent data collection, and data analysis were performed in a 7500 Fast real-time PCR System (Applied Biosystems), according to the manufacturer's instructions.

Analytical specificity and sensitivity

To investigate whether the probes and respective flanking primers detect their specific targets, DNA from *B. burgdorferi* s.l., from other spirochetes (*Leptospira interrogans* and *Treponema paliddum*) and from others tick-borne pathogens (*Theileria* sp. and *Babesia* sp.), were used as templates in real time PCR.

To estimate the detection threshold of the assays (analytical sensitivity), individually and as duplex and tetraplex real-time PCR, a standard curve was constructed using 10-fold serial dilutions of DNA extracted from *B. valaisiana*, *B. bavariensis*, *B. burgdorferi* s.s., *B. afzelii*, *B. garinii* and *B. lusitaniae* strains. The DNA dilutions for *B. burgdorferi* s.l. corresponded to 10 – 10^6 genome equivalents (GE), according to the National Reference Centre for Borrelia (NRZ units: 50fg/µl=10GE). For each strain and concentration the PCR assays were performed in triplicate. The end-point corresponded to the dilution at which the assay could detect the respective DNA targets in all three replicates.

To obtain an indication if the real-time PCR assays can be used with clinical samples, i.e., material of patients infected by *B. burgdorferi* s.l., sera were mixed with a 10-fold serial dilution of *B. burgdorferi* s.l. DNA (mixture of *B. burgdorferi* s.s., *B. afzelii*, *B. garinii* and *B. lusitaniae*) ranging from 10 to 10^6 NRZ units of GE. *Borrelia* DNA was re-extracted from this mixture using Gentra Puregene commercial kit from QIAGEN®, according to the manufacturer's protocol. These patient samples experimentally inoculated were screened accordingly with the real time PCR assay.

B. burgdorferi s.l. reference strains

B. burgdorferi s.s. (B31), *B. afzelii* (PGau), *B. garinii* (PBi) isolated from Japan, *B. lusitaniae* (PoHL1), *B. bavariensis* (PBi) and *B. valaisiana* (VS116) fresh cultures from the laboratory of Leptospirosis and Lyme Borreliosis Group from Instituto de Higiene e Medicina Tropical (IHMT)/UNL, were cultured in BSK-H medium, incubated at 34ºC and observed with a dark-field microscope every other day. When the cultures archived the logarithmic phase, the bacteria were harvested by centrifugation at a speed of 14000*g*, and genomic DNA extraction was performed with Gentra Puregene commercial kit from QIAGEN®, according to the manufacturer's protocol. After extraction the DNA concentration and purity from each *B. burgdorferi* s.l. genospecies were estimated by measuring the absorbance at 260 nm (A260) and by A260/A280 and A260/A230 ratios, using a NanoDrop 1000 spectrophotometer (NanoDrop™). The DNA concentration was adjusted to 10^6 GE for the six *B. burgdorferi* s.l. genospecies and dilutions from 10 to 10^6 GE were prepared.

Evaluation of real-time PCR with field-collected ticks and clinical samples

For the evaluation of the two-step multiplex real-time PCR identification assay, a panel of DNA samples, previously positive or negative for *B. burgdorferi* s.l., were obtained from (i) sera (n= 20) and cerebrospinal fluid (CSF) (n= 10) samples from human patients available at Leptospirosis and Lyme Borreliosis Group (from 2012 to 2015) and (ii) questing nymphs and adults of *Ixodes ricinus* species (n=50) collected across Portugal in previous studies (Nunes et

al., 2015; Nunes et al., 2016). The presence of *B. burgdorferi* s.l. DNA was formerly evaluated by two nested-PCR targeting the genes encoding the 5S-23S intergenic spacer region (Rijpkema et al., 1995) and the *flaB* gene (Wodecka et al., 2010). Nested-PCR amplification products from tick samples, were also previously sequenced for the identification of *B. burgdorferi* s.l. genospecies.

Statistical analysis

For measuring the agreement between the results of the routinely performed molecular identification of clinical and tick samples, and the real-time PCR assay, kappa coefficient was used. This coefficient, with confidence intervals, was determined with BioEstat 5.0.

Results

Analytical specificity and sensitivity

The two real-time PCR assays only detected *B. burgdorferi* s.l. DNA and did not produce any non-specific amplification products in repeated experiments. In addition, there were no false positives due to cross-reaction between fluorophore signals within each assay.

For the evaluation of the sensitivity, the assay was tested using DNA extracted from *B. burgdorferi* s.l. cultures as template. In the first step, dilutions from 10 to 10^6 GE of *B. burgdorferi* s.l. genospecies DNA were tested one by one and in a mixture with the six genospecies DNA, along with the internal controls; in the second step dilutions from 10 to 10^6 GE of DNA from *B. burgdorferi* s.s., *B. afzelii*, *B. garinii* and *B. lusitaniae* were tested individually and in tetraplex (Figure 3).

The results of the analytical sensitivity were as follows:

The first duplex reaction could detect the presence of *B. burgdorferi* s.l. until the dilution containing 50 fg/µL = 10 GE of DNA template, regardless the genospecies tested. The standard curve for DNA mixture showed a correlation coefficient (R^2) of 0.98 and a slope of – 3.2, indicating a good efficiency (106%) of PCR amplification (Figure 2).

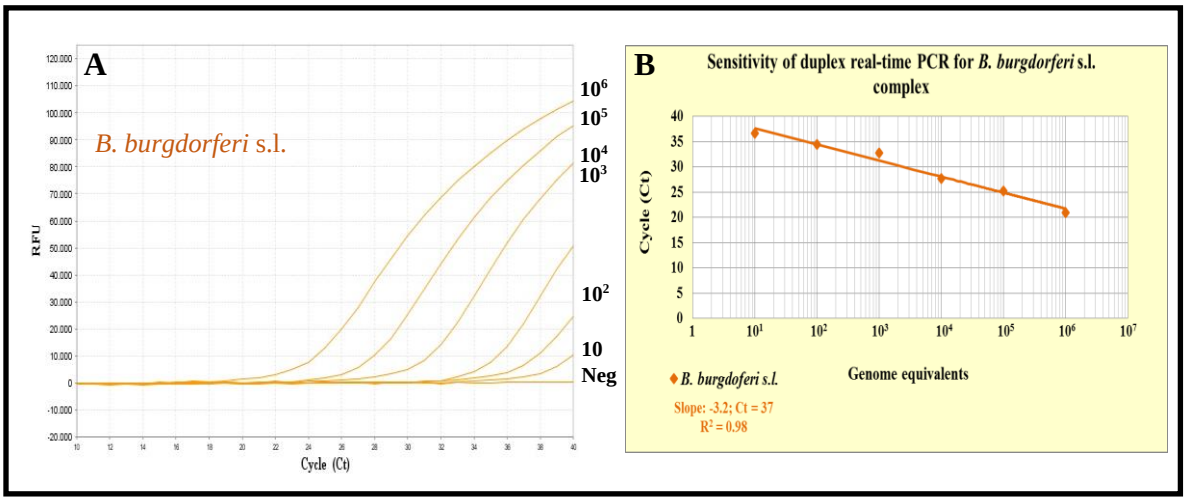

Figure 2 – Illustration of the duplex real-time PCR amplification curve obtained for each DNA concentration. (A) *B. burgdorferi* s.l. DNA dilutions from 10 to 10^6 GE; (B) respective linear relationship between the logarithm of the starting concentration of DNA and the amplification Ct values; Neg – real-time PCR negative control using DNase free water as template; Ct - interception in the minimum threshold (10 GE); RFU - Relative Fluorescence Units.

For the tetraplex reaction targeting the *flaB* gene of the four genospecies of *B. burgdorferi* s.l., when each probe was individually tested, the detection limit was 50 fg/µl = 10 GE for *B. afzelii* (Ct ≈ 37), *B. garinii* (Ct ≈ 37) and *B. lusitaniae* (Ct ≈ 37) and 0.5pg/µl = 10^2 GE for *B. burgdorferi* s.s. (Ct ≈ 36), (Figure 3 A,B,C and D); when tested in tetraplex the detection limit was 0.5pg/µl for *B. afzelii* (Ct ≈ 32), *B. garinii* (Ct ≈ 35), *B. lusitaniae* (Ct ≈ 35) and *B. burgdorferi* s.s. (Ct ≈ 35), (Figure 3E). The standard curves for the tetraplex reaction showed correlation coefficients (R^2) ranging from 0.929 to 0.997 and slopes of -2.296 to -3.377 (Figure 3F).

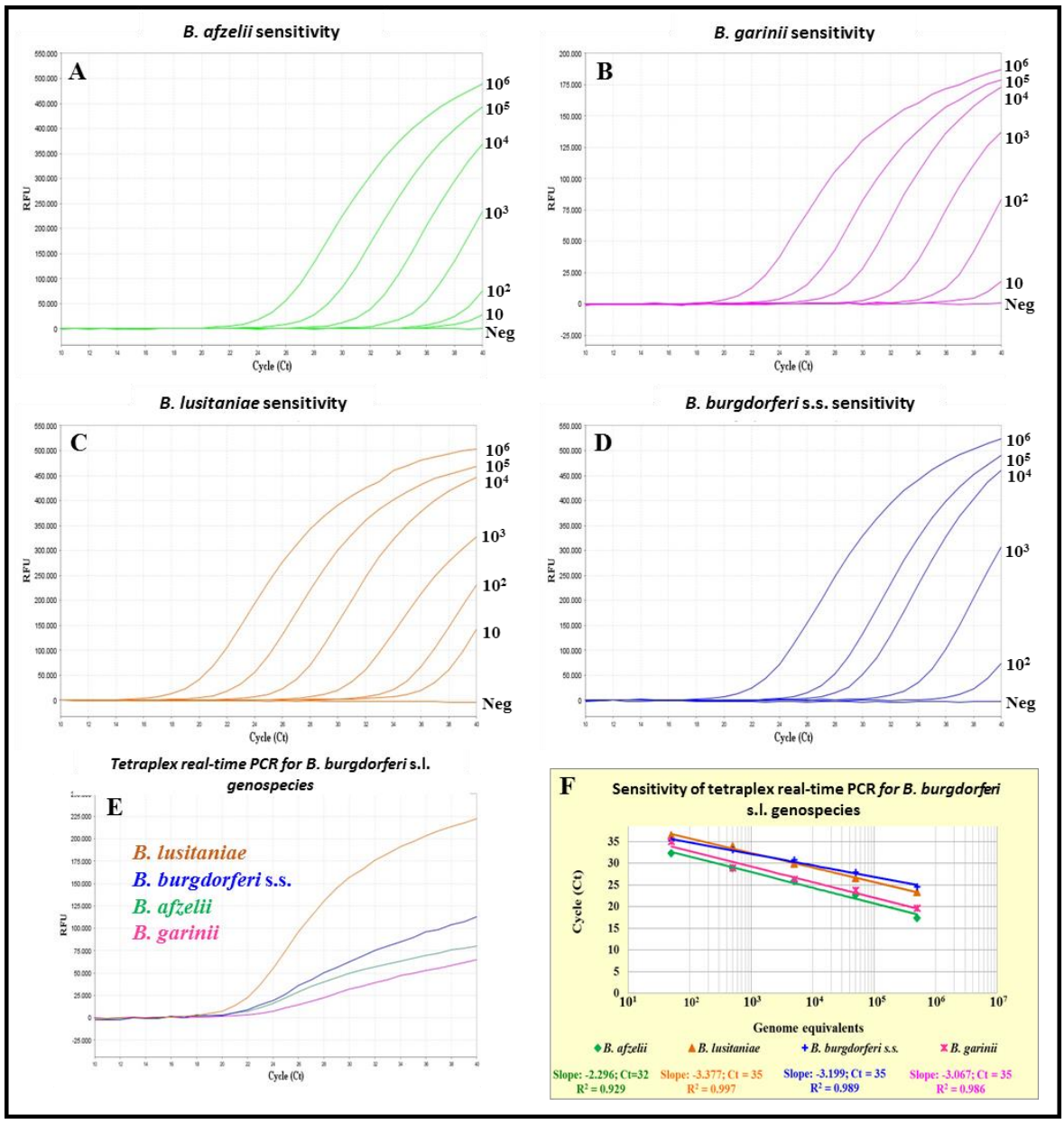

Figure 3 - Illustration of the tetraplex real-time PCR amplification curves obtained for each probe individually (A, B, C and D) and in tetraplex (E) for each DNA concentration; and respective linear relationship between the logarithm of the starting concentration of DNA and the amplification Ct values (F). A – real-time PCR for *B. afzelii* (dilutions from 10 – 10^6 GE); B – real-time PCR for *B. garinii* (dilutions from 10^6 – 10 GE); C – real-time PCR for *B. lusitaniae* (dilutions from 10^6 – 10 GE); D – real-time PCR for *B. burgdorferi* s.s. (dilutions from 10^6 – 10^2 GE); E – tetraplex real-time PCR for the dilution of 10^6 GE; Neg – real-time PCR negative control using DNase free water as template; Ct - interception in the minimum threshold; RFU - Relative Fluorescence Units.

Experimental inoculated serum samples

Screening of dilution series of DNA purified from patient's sera samples spiked with *B. burgdorferi* s.l. DNA, revealed no differences in the sensitivity of the duplex assay for the used material, since it was possible to obtained amplification signal until the dilution of 10 GE with a Ct value of 35. Regarding the tetraplex assay, the four genospecies tested did not show major differences in the sensitivity, since it was possible to obtain amplification signal until to dilution of 10^2 GE with Ct values of: 32 for *B. afzelii* and *B. burgdorferi* s.s., 35 to *B. garinii*, and 34 for *B. lusitaniae*.

Evaluation of real-time PCR with field-collected ticks and clinical samples

From the 50 tick samples tested, 24 (48%) previously positive for the two nested-PCR's, were also positive for the duplex real-time PCR, however, for the tetraplex real-time PCR just 23 (46%, test k= 0.96) samples were positive (Table 2). The genospecies of *B. burgdorferi* s.l. identified in this assay, were in agreement with the previously sequencing results (Table 2).
Concerning the clinical samples tested (n=30), from the 11 samples (40%) previously positive for the two nested-PCR, 11 (37%,), were also positive for the duplex real-time PCR, but only three (10%), were positive for the tetraplex assay, where the genospecies obtained were identified as: *B. afzelii*; *B. garinii* and B. *lusitaniae* (Table 2) and the K value was 0.93 and 0.32 for the duplex and tetraplex real-time PCR, respectively.

Table 2 – Comparison of duplex and tetraplex real-time PCR's positive samples with results from previous sequencing for tick samples.

***I. ricinus* samples (n=50)**			
Samples (Nested-PCR's positive or negative)	**Sequencing results**	**Duplex real – time PCR**	**Tetraplex real-time PCR**
1	*B. afzelii*	1 positive (Ct ≈ 17)	1 *B. afzelii* (Ct ≈ 21)
3	*B. burgdorferi* s.s.	3 positives	2 *B. burgdorferi* s.s. (Ct ≈ 33; Ct ≈ 36) 1 negative
8	*B. garinii*	8 positives (Ct ≈ 17 to Ct ≈ 19)	8 *B. garinii* (Ct ≈ 16 to Ct ≈ 33)
12	*B. lusitaniae*	12 positives (Ct ≈ 18 to Ct ≈ 26)	12 *B. lusitaniae* (Ct ≈ 20 to Ct ≈ 35)
26 negatives	-------	26 negatives	26 negatives
Clinical samples (n= 30)			
Samples (Nested-PCR's positive or negative)	**Sequencing results**	**Duplex real – time PCR**	**Tetraplex real-time PCR**
5 sera 6 CSF (positive)	------- -------	11 positives (Ct ≈ 17 to Ct ≈ 36)	1 serum as *B. afzelii* (Ct ≈ 29) 1 serum as B. *garinii* (Ct ≈ 30) 1 serum as *B. lusitaniae* (Ct ≈ 32) 8 negatives (2 sera; 6 CSF)
15 sera; 4 CSF (negatives)	-------	19 negatives	19 negatives

Discussion

According to European Center for disease Prevention and Control (ECDC) the diagnosis of *Borrelia* spp. infection should be based mainly on clinical symptoms, the patient's medical history and an evaluation of the risk of exposure to infected ticks, along with diagnostic tests including the assessment of antibodies to *Borrelia* spp. class IgM and IgG (Bil-Lula et al., 2015). However, the serologic tests based in antibodies search have some problems concerning the large amount of false negative results, probably due to the "window period" in which IgM antibodies are not yet produced. Consequently, molecular approaches as real-time PCR assays would be helpful in testing patients early in the disease, before an antibody response develops, and in patients presenting non classic symptoms.

It is also known that different *Borrelia* genospecies are associated with diverse biological origins (*B. afzelii* with small mammals, *B. garinii* with birds and *B. lusitaniae* with lizards)

(Kurtenbach et al., 2002; Mannelli et al., 2012) , different clinical manifestations (*B. garinii* with neurological manifestation, *B. afzelii* with skin manifestations) (van Dam et al., 1993), severity of disease (*B. burgdorferi* s.s. is more severe than *B. afzelii*) (Jungnick et al., 2015), and geographic distribution of species (*B. afzelii*, *B. garinii* and *B. lusitaniae* in Europe and *B. burgdorferi* s.s. in North America) (van Dam et al., 1993; Stanek et al., 2002; Stanek & Strle, 2003) Consequently, having the capacity of identifying the *Borrelia* genospecies involved in an infection, whether in the vector or in the host, is becoming increasingly important since it also provides information on the ecological characteristics of individual species, allows a better prognosis and treatment strategy, and is needed for genetic analysis (Mukhacheva & Kovalev, 2014).

Quantitative real-time PCR for direct molecular detection and quantification of pathogens is a widely used technology nowadays for clinical application, being also valuable for confirming a diagnosis based on less clear manifestations of LD or for investigating controversial disease syndromes attributed to infection with *B. burgdorferi* s.l.. Several real time PCR assays for the detection of *B. burgdorferi* s.l. have been reported previously, however, few have included an internal control (Germer et al., 1999; Gooskens et al., 2006), nor has a quantitative tetraplex PCR study for four of the most prevalent *Borrelia* genospecies in Europe been published to date.

Therefore, in this study, we present a combined multiplex *TaqMan* real-time PCR strategy to infer the presence of *B. burgdorferi* s.l. genospecies in clinical and vector samples. In the first step we evaluated if a sample is infected with *Borrelia burgdorferi* s.l. by targeting the *flaB* gene. The *flaB* gene encodes a 41-kDa flagellin protein and is located on a single-copy in the linear chromosome (Wang et al., 1999). In this step the inclusion of an internal control allowed successful DNA extraction to be monitored. The second step identifies simultaneously four of the most prevalent genospecies of *B. burgdorferi* s.l. in Europe, namely *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s. and *B. lusitaniae*. Although it targets the same gene as the previous step, the primers were designed in a different more variable region, resulting in a fragment with sufficient polymorphisms that allowed to design the four specific probes for each genospecies.

In the duplex real-time PCR, DNA from each *B. burgdorferi* s.l. genospecies, available at Leptospirosis and Lyme Borreliosis laboratory, IHMT/UNL, was tested individually and in a

mixture containing the same DNA concentration of the six genospecies (*B. afzelii, B. garinii, B. lusitaniae, B. burgdorferi* s.s., *B. valaisiana and B. bavariensis*). Whether the DNA is tested individually or simultaneously, all genospecies showed very good reactivity, since no differences were obtained regarding the sensitivity (10 GE). Similar results were obtained for *B. burgdorferi* s.l. in previously studies, whose sensitivity's range from 1 to 10 GE (Gooskens et al., 2006; O'Rourke et al., 2013; Venczel et al., 2015). Furthermore, the assay is highly specific, as it failed to detect the *flaB* gene of other microbial species.

For the tetraplex assay the sensitivity of the assay decreases from 10 GE to 10^2 GE for *B. garinii*, *B. afzelii* and *B. lusitaniae* but remains the same for *B. burgdorferi* s.s., when the four probes are tested simultaneously. This loss of sensitivity is normal when passing from a singleplex to a multiplex real-time PCR, and is related with the competition between the targets, since we are using the same pair of primers for the four targets, being the differences only found in the probes.

When the assay was applied to field tick samples, the duplex step presented a very good performance, since it gave the same results regarding the positive and negative samples obtained previously by the two nested-PCR targeting the *flaB* gene and the IGS region. Also for the tetraplex assay only one of the positive tick samples yielded a negative result, probably due to the detection limit of the assay, since it is lower than the detection limit of the duplex assay. Moreover, the *B. burgdorferi* s.l. genospecies identified by the tetraplex were 100% equal as those obtained from the sequencing results. The assays sensitivity is crucial when analyzing tick samples, since they have the capacity of harboring complex microbial populations (Tveten & Sjåstad, 2011). The diverse bacterial content in ticks could be responsible for a low amount of *Borrelia* spirochetes, due to the natural size of the ticks, and also to the environmental competition between bacterial species (Hibbing et al., 2010).

Regarding the testing of clinical samples (sera and CSF) with the duplex assay, a 100% agreement was achieved with the results previously obtained with the nested-PCR's protocols. However, in the tetraplex assay only three samples were positive, and identified as *B. afzelii*, *B. garinii* and *B. lusitaniae*; the remainder eight nested-PCR-positive samples were negative with this assay, including all the CSF samples tested. This fact is related with the loss of sensitivity when the four probes are used simultaneously. However, previously studies showed

that *Borrelia* counts in CSF are very low, around 20 bacteria per 100µL CSF lysed (Nocton et al., 1996; Schwaiger et al., 2001; Gooskens et al., 2006; Bil-Lula et al., 2015), which is below the tetraplex sensitivity, but in the same baseline of the duplex assay developed in our study.

Numerous PCR assays have been described for the detection of *B. burgdorferi* s.l. DNA in CSF, but the sensitivities varied from 12% to 100% (Keller et al., 1992; Lebech & Hansen, 1992; Eiffert et al., 1995; Nocton et al., 1996; Lebech et al., 2000; Schwaiger et al., 2001). These results are difficult to interpret because of the use of small sample sizes, the selection differences of clinical specimens, the testing of poorly defined patient categories, and the frequent lack of an internal control to monitor PCR inhibition. In our case, both negative and positive controls and an internal control (mammals-β-actin gene) were included in each run to determine whether or not inhibitory substances were present in the patient's clinical sample, or whether false positive results could appear during amplifications. Thus the possibility of low test sensitivity due to the presence of PCR inhibitors in CSF samples is excluded. However, for a better evaluation of this combined multiplex *TaqMan* real-time PCR, further investigation using other clinical samples is required.

In conclusion, this two-step multiplex *TaqMan* real-time PCR assay targeting the *flaB* locus, proved to be an efficient method when screening for *Borrelia* infection in tick samples, and a promising tool for early diagnostic purposes on clinical samples. Moreover, the ability to detect four of the most prevalent *B. burgdorferi* s.l. genospecies in Europe, in a single-run is a time-saving factor, and cost reduction when compared with the conventional PCR and sequencing methods.

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5.2 - Development of a Loop Mediated Isothermal Amplification (LAMP) assay for the detection of *Borrelia burgdorferi* s.l. genospecies DNA in tick samples

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Abstract

Lyme disease (LD) is a zoonosis caused by spirochetes belonging to *Borrelia burgdorferi* sensu lato (*B. burgdorferi* s.l.) complex that are transmitted by hard-ticks from *Ixodes* genus, namely *Ixodes ricinus* in Europe. There are at least 20 described *B. burgdorferi* s.l. genospecies, being the most prevalent in Europe *Borrelia burgdorferi* sensu stricto (s.s), *B. garinii*, *B. afzelii*, *B. valaisiana* and *B. lusitaniae*. Each of these genospecies presents tropisms to different organs and systems of the host (skin, articulations, neurologic and nervous systems). Therefore, it is important to have sensitive and specific diagnostic tools allowing the identification of the different genospecies in an early phase of infection.

The aim of this study was to develop and evaluate two duplex LAMP assays (dLAMP) based on *flaB* gene, combined with a Lateral Flow Device (LFD) technology, to implement a rapid, simple and sensitive assay, for the identification of four of the most prevalent genospecies of B. *burgdorferi* s.l. complex in Europe and Portugal, namely *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s. and *B. lusitaniae*.

A set of primers was designed for each *Borrelia* genospecies using the software "PrimerExplorer V4", and specificity of each set was tested. LAMP primer sets showed no specificity to their targeted genospecies, but all sets amplified only *B. burgdorferi* s.l. complex DNA templates. The sensitivity was assessed for *B. lusitaniae* primers set, which amplified the four *Borrelia* genospecies, and compared with those of two nested-PCRs targeting the intergenic spacer region (IGS) and the *flaB* gene, and one real-time PCR also targeting the *flaB* gene. Sensitivities varied depending on the genospecies tested, ranging from 2.5 pg/µl for *B. lusitaniae* to 2500 pg/µl for *B. garinii* DNA. Sensitivities of both nested-PCRs and the real-time PCR were lower than those of LAMP, ranging from 0.05 to 5pg/µl.

It was not possible to achieve the initial goal of this study. However, the sensitivity of the LAMP technique was similar to those of the others molecular approaches when using *B. lusitaniae* primers set with *B. lusitaniae* DNA template. Although, being more difficult to optimize and to control the occurrence of false-positive results, LAMP has the advantage of occurring in a single tube in less than one hour and by using a simple water bath, where the

amplification products can be observed by naked eye. Therefore, this technique may have its role as a tool in low-resource laboratories for the diagnosis of LD in an early stage of infection.

Introduction

Borrelia burgdorferi sensu lato (*B. burgdorferi* s.l.) complex is a group of several genospecies of spirochetes responsible for Lyme disease (LD), the world's fastest growing vector-borne zoonotic disease with cases reported in over 60 countries and endemic foci in North America, Europe, and Asia (WHO, 2013). This complex is represented by 20 genospecies, several of which can cause LD in humans. These genospecies vary in their geographic distribution, host specificity and ability to cause disease in humans. Clinically the different pathogenic *Borrelia* spp. are of interest as they have been associated with different disease symptoms which may be observed in the late stages of the condition (Margos et al., 2011). In Europe LD is mostly associated to one of three genospecies: *B. burgdorferi* s.s., *B. afzelii* and *B. garinii* (Assous et al., 1993; van Dam et al., 1993; Richter et al., 2004). *B burgdorferi* s.s. is normally associated with arthritis, *B. garinii* with neurological effects (musculoskeletal and nervous systems), and *B. afzelii* with skin complications, like Acrodermatitis Chronica Atrophicans (ACA) (van Dam et al., 1993).

The laboratory diagnosis is based mainly on serological and molecular biology methods. Serological methods includes screening tests such as enzyme-linked immunosorbent assays (ELISA), indirect immunofluorescence assays (IFA), and confirmation tests such as Western blot (Robertson et al., 2000; Steere et al., 2008; Hinterseher et al., 2012; Liu et al., 2013). Regarding the molecular approaches, several PCR-based methods have been developed to detect *B. burgdorferi* s.l. DNA, such as conventional PCR, nested PCR, and real-time PCR based in specific gene detection, for several markers such as, *ospA*, *rrs*, *rrf-rrl* intergenic spacer, *groEL*, *recA*, *hbb*, *fla*, and others (Picken et al., 1996; Priem et al., 1997; Aguero-Rosenfeld et al., 2005; Kondrusik et al., 2007; Wang et al., 2010; Wodecka et al., 2010; Wodecka 2011; de Leeuw et al., 2014). Nevertheless, most of these molecular approaches have several disadvantages: time-consuming, variable sensitivity and requires specific

expensive instruments for the amplification reaction, which is difficult in low incoming countries. Therefore, rapid, simple, low-cost, and effective diagnostic methods are urgently required.

Loop-mediated isothermal amplification (LAMP) was first reported in 2000 by Notomi (Notomi, 2000), and since then the number of studies performed for the detection of a wide range of viruses, parasites and bacteria, using this technology is increasing every year (Pooja et al., 2014; Fallahi et al., 2015; Gao et al., 2015; Jung et al., 2015; Wang et al., 2015a). The principle of this technology relies on an autocycling strand displacement DNA synthesis by a DNA polymerase with high strand displacement activity (*Bst*), superseding thermal denaturation steps (Notomi, 2000). The research and development efforts on LAMP technology, in the last years, have been focused on its practical application in clinical settings including the improvement of existing assays. The most distinctive characteristics of LAMP are its simplicity and its rapidity, representing its major advantages over the PCR-based technique (Mori et al., 2013; Notomi et al., 2015). Because this technology is an isothermal method, LAMP can be performed just by using an inexpensive heater like a block heater or a water bath, allowing, this way, for LAMP to be conducted in any time and any setting.

Moreover, besides the conventional methods to analyze LAMP products, such as agarose gel electrophoresis, visual inspection of the increase turbidity, or colour change of the reaction mixture (Mori et al., 2001), this technology can be attached to other devices such as lateral-flow devices (LFDs) for the detection of labels incorporated into the amplification products.

LFD tests have a number of advantages for use in the field, and specific LFD immunoassays have been particularly successful in areas of point-of-care and on-site testing (Assadollahi et al., 2009; Baumert & Tran, 2015; Sajid et al., 2015). There are LFD generic test commercially available, like chromatographic strips that can detect biotin-labelled DNA fragments hybridized with complementary FITC-labelled probes. FITC is detected in the strips by the formation of complexes with gold-conjugated anti-FITC antibodies.

The aim of this study was to develop and evaluate two duplex LAMP assays (dLAMP) based on *flaB* gene, combined with a LFD technology, to implement a rapid, simple and sensitive

assay for the identification of four of the most prevalent genospecies of *B. burgdorferi* s.l. complex (*B. afzelii, B. garinii, B. burgdorferi* sensu stricto (s.s.) and *B. lusitaniae*).

Material and methods

Borrelia burgdorferi s.l. *strains*

B. burgdorferi s.s. (B31), *B. afzelii* (PGau), *B. garinii* (PBi), and *B. lusitaniae* (PoHL1), fresh cultures available at the Group of Leptospirosis and Lyme Borreliosis (GLBL) from Instituto de Higiene e Medicina Tropical (IHMT)/UNL, were incubated for one week in BSK-H medium at 34ºC. The growth was detected by examining the culture using a dark-field microscope and collection of the spirochetes was done in the log-phase (approximately 10^8 – 10^9 bacteria/ml).

DNA extraction

Total genomic DNA extraction from *B. burgdorferi* s.l. cultures was performed with Gentra Puregene commercial kit from QIAGEN®, according to the manufacturer's protocol. After extraction the DNA concentration and purity were estimated by measuring the absorbance at 260 nm (A260) and by A260⁄A280 and A260/A230 ratios, using a NanoDrop 1000 spectrophotometer (NanoDrop™). The DNA concentration was adjusted to 10^6 genome equivalents (GE) ≈ 5 ng/µl of DNA, according to the National Reference Centre for Borrelia (NRZ units), for the four *B. burgdorferi* s.l. genospecies and dilutions from 10 to 10^6 GE were prepared.

Design of LAMP primers

A multiple alignment of flagellin (*flaB*) sequences retrieved from GenBank was created in Mega 6 (Kumar et al., 2008), and a Flagellin sequence consensus for each genospecies was selected: *B. burgdorferi* s.s. (accession number X15661.1), *B. garinii* (accession number DQ650333.1), *B. afzelii* (accession number DQ016619.1), and *B. lusitaniae* (accession

number D82856.1). The primer sets for each LAMP were designed using PrimerExplorer V4 software (http:// primerexplorer.jp/elamp4.0.0/index.html). This primer set included two outer primers (F3 and B3) and two inner primers (FIP and BIP) for each *Borrelia* genospecies. To reduce the reaction times, we also designed two loop primers (LF and LB) for each species (Figure 1). The primer sequences are shown in Table 1. All primers were synthesized and HPLC-purified by StabVida Lda, Portugal.

Table 1 – LAMP primers designed targeting the four *B. burgdorferi* s.l. genospecies.

Genospecies	Primers	Sequence
B. afzelii	Forward Inner Primer (FIPBa)	5'-TTCATCTTGATTTGCTCCCACATG-AACACACCAGCATCACTTTC-3'
	Backward Inner Primer (BIPBa)	5'-AGCTAATGTTGCAAATCTTTTTGCT-CTTCTTCTTGAGCACCCTC-3'
	Forward 3 (F3Ba)	5'-AGCTGAAGAGCTTGGAATG-3'
	Backward 3 (B3Ba)	5'-TTGAGTAGGTGCTGTAGC-3'
	Loop Forward (LFBa)	5'-AGTCCAAGAAGCTTGAGATCCT-3'
	Loop Backward (LBBa)	5'-GGAGCTCAAGCTGCTCAGGC-3'
***B. burgdorferi* s.s.**	Forward Inner Primer (FIPBb)	5'- GGTTGCTCCAACATGAACTCTTAA-AACACACCAGCATCACTTTC - 3'
	Backward Inner Primer (BIPBb)	5'-GCAGCTAATGTTGCAAATCTTTT-CTGAACACCCTCTTGAACCG-3'
	Forward 3 (F3Bb)	5'-AGCTGAAGAGCTTGGAATG-3'
	Backward 3 (B3Bb)	5'-GTTGAGCTCCTTCCTGTT-3'
	Loop Forward (LFBb)	5'-CCAAGACGCTTGAGACCCT-3'
	Loop Backward (LBBb)	5´-GAGGGAGCTCAAACTGCTCAGG-3´
B. garinii	Forward Inner Primer (FIPBg)	5'-TCACCAGAGAATAGATTTGCAA-CATGAGCAAATCAAGATGAAGCG-3'
	Backward Inner Primer (BIPBg)	5'-ACCTGTTCAAGAAGGAGCTCA-AATTAACTCCACCCTGAGAA-3'
	Forward 3 (F3Bg)	5'-TCTTGGACCTTAAGAGTTCA-3'
	Backward 3 (B3Bg)	5'-GATGTATTAGCGTCAACTGTG-3'
	Loop Forward (LFBg)	5'-TTAAGGTCCAAGAAGCTTGAGATC-3'
	Loop Backward (LBBg)	5'-TCTGGTGAAGGAGCTCAGGCT-3'
B. lusitaniae	Forward Inner Primer (FIPBl)	5'-ATCTTGATTTGCTCCCACATG-AACTCACCAGCATCACTTTCAGG-3'
	Backward Inner Primer (BIPBl)	5'-ATGTTGCAAATCTGTTTTCTGGT - GGCTCCTTCTTGTTGAACAC-3'
	Forward 3 (F3Bl)	5'-AGCTTGGAATGCAACCTG-3'
	Backward 3 (B3Bl)	5'-CTTGAGAAGGCGCTGTAG-3'
	Loop Forward (LFBl)	5'-CTCAAAGTCCAAGAAGCTTGAGAT-3'
	Loop Backward (LBBl)	5'-GGGAGCTCAAGTTGCTCAGACTG-3'

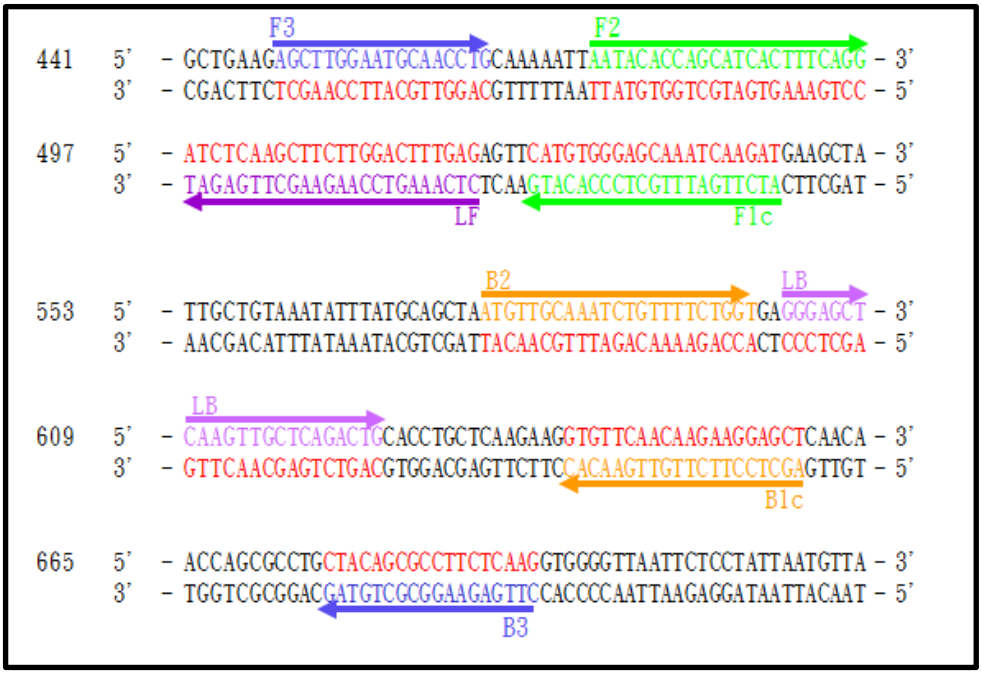

Figure 1 – Partial sequence of *flaB* gene for *B. lusitaniae*, and location of the complementary regions used to design LAMP primers [F3, B3, FIP (F1c-F2), BIP (B1c-B2)], including loop primers (LF, LB). Arrows indicate the direction of extension.

Optimization of LAMP reaction

Based on the initial conditions of the LAMP reaction adopted from Notomi et al., (2000), three Mg^{2+} concentrations (2 mM, 8mM, and 10 mM), three *Bst* DNA polymerase concentrations (2.0 U, 4.0 U, and 8.0 U), two concentration ratios between inner to outer primers (4:1 and 8:1) and two betaine concentrations (0.80 M and 1.0M) were tested in 10 µL reaction mixtures, while the concentrations of all the other components remained constant. The temperature of LAMP reaction was also optimized by testing four temperatures (66, 67, 68 and 69ºC), and also two incubation periods (45 and 60 min). These conditions were first tested only with *B. lusitaniae* LAMP assay, and after optimization the final

conditions were applied to the remaining three LAMP assays targeting *B. afzelii*, *B. garinii* and *B. burgdorferi* s.s..

Analytical specificity and sensitivity of LAMP assays

The specificity of the LAMP assays for detecting *B. burgdorferi* s.l. DNA was determined using genomic DNA of *B. burgdorferi* s.s. (B31), *B. afzelii* (PGau), *B. garinii* (PBi), and *B. lusitaniae* (PoHL1) reference strains, and DNA from other microorganisms namely other spirochetes like *Leptospira interrogans* (Serovar Icterohaemorrhagiae) and *Treponema pallidum*; and other tick-borne pathogens like *Theileria* sp. and *Babesia* sp..
The sensitivity of LAMP reaction was determined by using 10-fold serial dilutions of DNA extracted from *B. burgdorferi* s.l. cultures, corresponding to 10 – 10^6 GE.

Nested-PCR protocols and real-time PCR

The serial dilutions of *B. burgdorferi* s.l. cultures were also used to evaluate the sensitivity of two nested-PCR protocols and one real-time PCR, in order to compare them with the sensitivity of LAMP assays.

One of the nested-PCR targeted the intergenic spacer region (IGS), located between the 5S and 23S rRNA, using the 23SN1 and 23SC1 external primers (which amplify a 320 bp DNA fragment), and the 23SN2 and 5SC inner primers (which amplify a 280 bp DNA fragment), as described by Rijpkema et al., 1995. The second nested-PCR protocol used, targeted the flagellin gene (*flaB*) (Wodecka et al., 2010). This included a first amplification reaction based on the use of outer primers 123f and 905r (which amplify a 774 bp DNA fragment), with a second amplification step using the inner primers 220f and 824r (yielding an amplification product of 605 bp). PCR protocols were done in a separate vertical laminar flow bench using a different set of micropipettes, for PCR use-only as well filtered tips and sterilized material to ensure a contamination-free environment. *B. garinii* DNA was used as positive control and ultrapure water as negative control. Products were detected by electrophoresis in 1.5%

agarose gels stained with GreenSafe Premium (NZYTech), and visualized under UV light, using a Dolphin-1D Gel Image Analysis Software (Wealtec®) equipment.

Regarding the real-time PCR protocol, it consisted in a duplex reaction targeting the *flaB* gene for *B.burgdorferi* s.l. and the internal control 18S rDNA for hard-tick samples. This real-time PCR protocol was developed and optimized by our laboratory (*data submitted*). The PCR reaction was carried out in a total volume of 20 µl containing 1× SensiFAST™ (Bioline), 0.3 µM of each primer (F_*Bb*sl, R_*Bb*sl), 0.25 µM of each *TaqMan* probe (P_*Bb*sl), DNase free water (Bioline) and 2 µl of the extracted DNA template. The thermal cycling conditions were: 1 cycle at 95 °C for 1 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 45 s. Thermal cycling, fluorescent data collection, and data analysis were performed in a 7500 Fast real-time PCR System (Applied Biosystems), according to the manufacturer's instructions.

LAMP product detection

LAMP reaction was performed in a final volume of 25 µl, containing 1× LAMP buffer (BioLabs®), 8 mM $MgSO_4$, 1 M Betaine (Sigma®), 0.4 mM dNTP, 1.6 µM of FIP and BIP primers, 0.2 µM of F3 and B3 primers, 0.4 µM of LF and LB primers, 8 U *Bst* 2.0 WarmStart® DNA polymerase (New England BioLabs® Inc., USA) and 2.5 µl of extracted DNA. The reaction mixture was incubated in an automatic thermocycler (Mycylcer, BioRad) at 68ºC for 45 min, and then inactivated at 80 ºC for 5 min.

LAMP products were detected by: *i)* assessment of turbidity by the naked eye resulting from the magnesium pyrophosphate ($Mg_2P_2O_7$), precipitation (Figure 2A); *ii)* color change at naked eye and under UV by adding 1.0 µl of 1/10-diluted original SYBR Green I (Invitrogen™), where samples that turned yellowish green were considered positive, while those remained orange were assumed to be negative (Figure 2B1 and B2); iii) by UV transillumination (Dolphin-Doc Plus Gel Image system, Wealtec® equipment) in a 2.5% agarose gel following electrophoresis in Tris-Acetate-EDTA (TAE) buffer stained with 2µl of GreenSafe Premium (NZYTech) (Figure 2C).

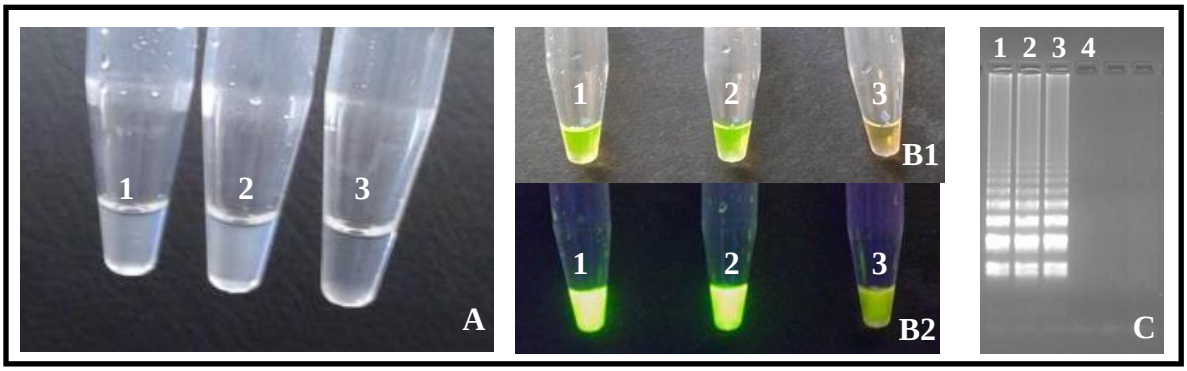

Figure 2 – LAMP products visualization, by naked eye through the observation of the turbidity (A), 1 and 2 - positive samples, 3 - negative control; by intercalating dyes like SYBR-Green under natural light (B1) and UV light (B2), 1 and 2 - positive samples, 3 negative control; by electrophoresis in a 2.5% agarose gel (C), 1,2 and 3 – positive samples, 4 – negative control.

Lateral Flow Device strips

For detection of LAMP products by LFD, the adequate FITC-labeled probe should be added to the LAMP products. The protocol include a first step of hybridization at 65 ºC for 5 min, and then 8 µl of the hybridized product is added to 100 µl assay buffer in a new tube. An LFD strip is dipped into the mixture for 2 min. Commercial universal LFD devices for the detection of labelled LAMP products were purchased from Milenia Biotec (HybriDetect 2T) and used according to the instructions of the manufacturer.

Statistical analysis

The results were analyzed by the Chi-square test using BioEstat 5.0. A difference was considered statistically significant at $P < 0.05$.

Results

Optimization of LAMP reaction

When the concentration of Mg^{2+} was evaluated, we observed that together with the increase of the temperature from 66 to 69ºC, the best concentration was 8 mM, since that at 2 mM the

detection limit of the reaction decreased and at 10 mM LAMP reaction was inhibited. Regarding the ratio of inner to outer primers more intense ladder-like bands were obtained at the concentration ratio 8:1 (Figure 3A), also the amplification improved obviously as the dosage of *Bst* DNA polymerase increased from 2.0 U to 8.0 U, as evidenced by brighter bands on agarose gels (Figure 3B). From the temperature and time of reaction variation, the detection limit was greater with higher temperatures (68ºC) and less time of reaction (45 min), since no unspecific results appeared (Figure 3C). Therefore the above results demonstrated that the optimized LAMP reaction consisted of using Mg^{2+} at a concentration of 8 mM, the ratio of inner to outer primers at 8:1, and the concentration of *Bst* DNA polymerase at 8.0 U. Also, LAMP assay was easy to conduct, although some measures concerning the prevention of contamination were necessary. Precautions such as changing gloves between every LAMP assay and different work areas for different parts of the experiment were taken into account.

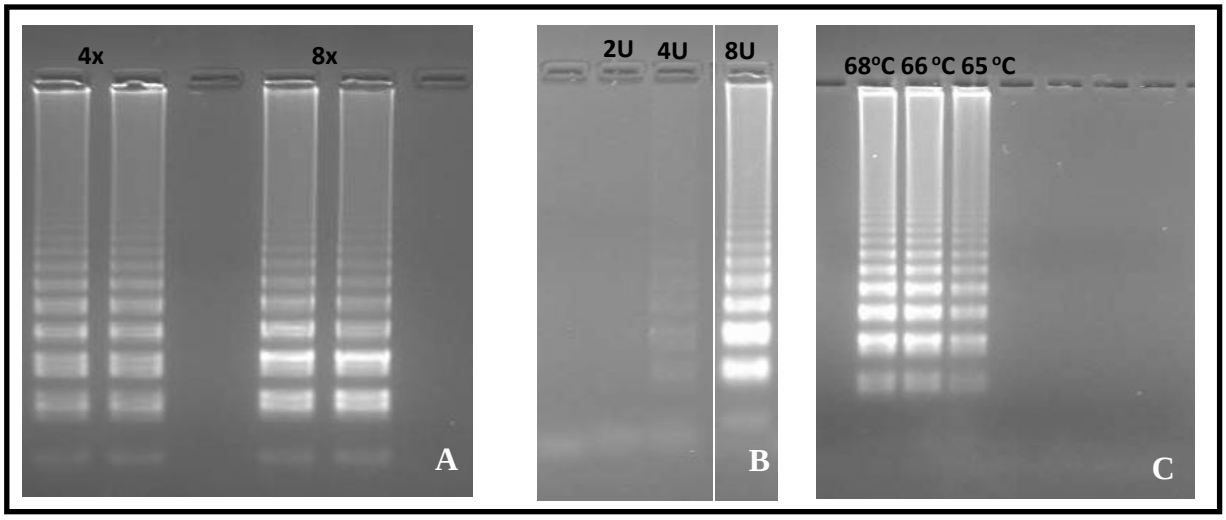

Figure 3 – LAMP assay optimization by testing: different FIP/BIP and F3/B3 concentrations ratio (A); different concentrations of *Bst* polymerase (B); and different temperatures of reaction (C).

Analytical specificity of LAMP assay

LAMP reaction specificity was evaluated and results showed that only the four *B. burgdorferi* s.l. genospecies produced a typical ladder of multiple bands on the agarose gel, while other DNA samples or negative control did not produce such bands. However, when each LAMP set was tested with DNA from the respective B. *burgdorferi* s.l. genospecies, the specificity obtained was not the expected, since each of the four sets amplified not only the respective *B. burgdorferi* s.l. genospecies but also one or more of the others *B. burgdorferi* s.l. genospecies. For example LAMP set for *B. garinii* amplified *B. garinii* DNA but also *B. afzelii* DNA, and LAMP set for *B. lusitaniae* amplified *B. lusitaniae* DNA but also the others three genospecies DNA, *B. garinii, B. burgdorferi* s.s. and *B. afzelii*. Therefore each LAMP set was specific for *B. burgdorferi* s.l. genera but not for each genospecies. This lack of specificity led us to continue the work with only the *B. lusitaniae* LAMP primers set since it was able to amplify DNA templates of all four genospecies. This way we decided to develop a LAMP assay targeting the four of the most prevalent species of *B. burgdorferi* s.l. complex.

Analytical sensitivity

Since there was no specificity of LAMP sets for each genospecies, the analytical sensitivity of LAMP reaction was evaluated only with the *B. lusitaniae* primers set, since it amplified the four most important genospecies. The results showed that the detection limit was 0.5 ng/µl ≈ 10^4 GE for *B. afzelii*, 2.5 ng/µl ≈ 10^3 GE for *B. garinii*, 1 ng/µl ≈ 10^3 GE for *B. burgdorferi* s.s. and 2.5 pg/µl ≈ 10^2 GE for *B. lusitaniae* (Figure 4).

Regarding the nested-PCR protocol for *flaB* gene, the detection limit was 5 pg/µl ≈10^3 GE for *B. afzelii*, 0.5 pg/µl ≈ 10^2 GE for *B. lusitaniae,* and 0.05 pg/µl ≈ 10 GE for *B. burgdorferi* s.s and *B. garinii*. The nested-PCR protocol for IGS, presented a detection limit of 5 pg/µL ≈10^3 GE for *B. afzelii*, *B. burgdorferi* s.s and *B. garinii*, and 0.5 pg/µl ≈10^2 GE for *B. lusitaniae*. These results suggest that LAMP sensitivity was similar to that of the IGS targeted nested-PCR protocol, except for *B. afzelii* which was lower.

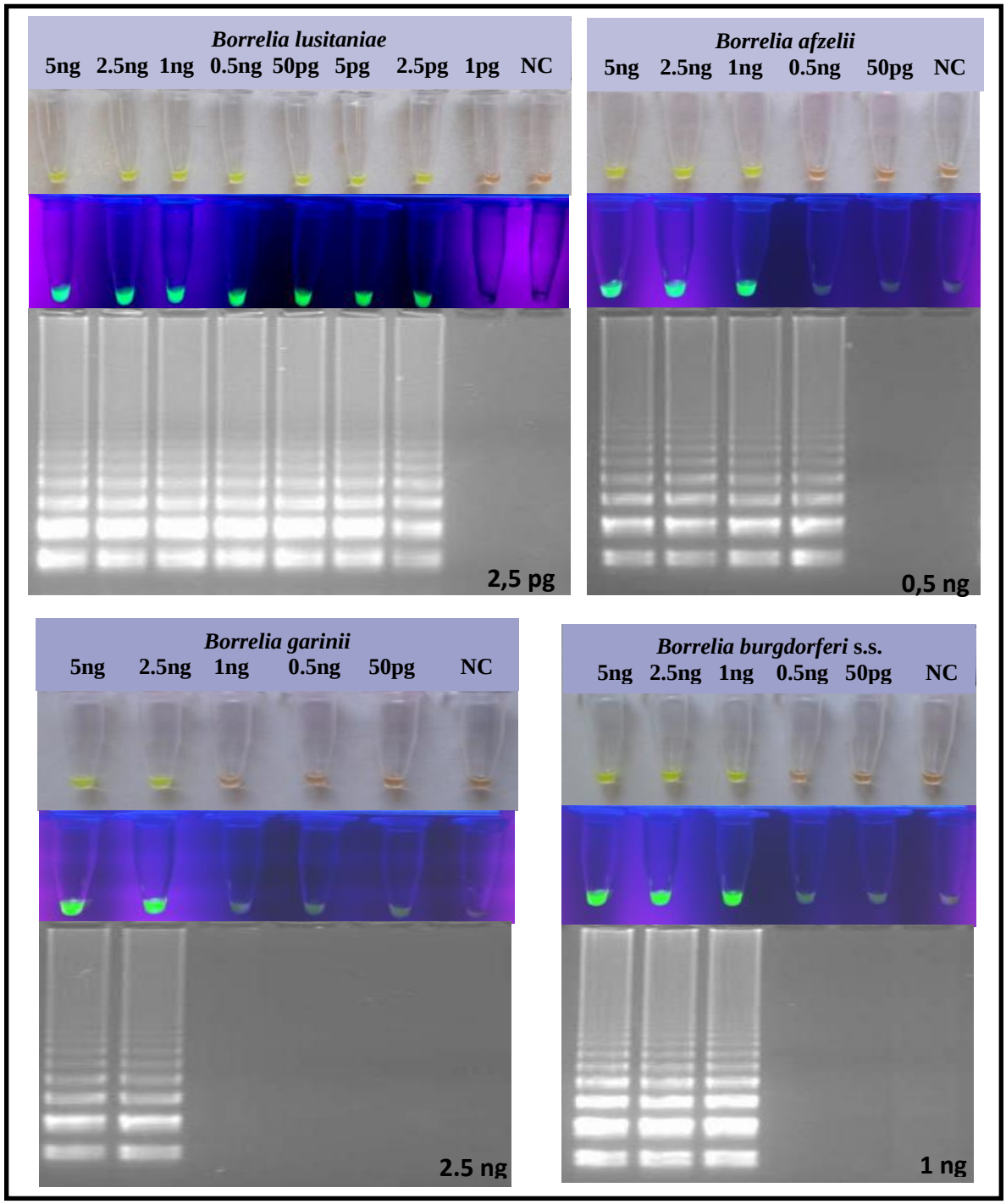

Figure 4 – Sensitivity optimization of LAMP assay with *B. lusitaniae* primers set for the four *B. burgdorferi* s.l. genospecies DNA serial dilutions, and visualization of amplification products by adding SYBR-Green under natural and UV light, and by electrophoresis in agarose gel.

Regarding the *flaB* targeted nested-PCR protocol, LAMP reaction sensitivity was the same only for *B. lusitaniae* genospecies, and lower for the remaining three genospecies (Table 2). The real-time PCR reaction could detect the presence of *B. burgdorferi* s.l. genospecies until the dilution containing 0.05 pg/µl = 10 GE (Ct ≈ 34) of DNA template, regardless the genospecies tested, presenting a higher sensitivity when compared to LAMP reaction (Table 2). Due to the obtained results, no statistical analysis was performed, until further improvements in LAMP technique.

Table 2 – Sensitivities obtained for the detection of *B. burgdorferi* s.l. genospecies with different molecular approaches.

	Nested-PCR (IGS)	Nested-PCR (*flaB* gene)	Real-time PCR (*flaB* gene)	LAMP (*flaB* gene)
Borrelia afzelii	5 pg/µl	5 pg/µl	0.05 pg/µl	500 pg/µl
***Borrelia burgdorferi* s.s.**	5 pg/µl	0.05 pg/µl		1000 pg/µl
Borrelia garinii	5 pg/µl	0.05 pg/µl		2500 pg/µl
Borrelia lusitaniae	0.5 pg/µl	0.5 pg/µl		2.5 pg/µl

Discussion

In Europe the most prevalent *B. burgdorferi* s.l. genospecies are *B. garinii*, *B. afzelii*, *B. burgdorferi* s.s. and *B. valaisiana*, however, in Portugal, *B. lusitaniae* also presents a high prevalence at the vector level (Norte et al., 2015). It is known that each one of these genospecies are responsible for different symptoms, due to their tropism to certain organs and systems of the host. In the laboratorial diagnosis the Gold Standard is mainly based in the detection of *B. burgdorferi* s.l. antibodies in biological samples, however, this approach is only possible to perform within two to three weeks after the infection, when the immune system is able to produce the specific antibodies, reaching a peak between the six and eight week for IgM and four to six months for IgG (Alby & Capraro, 2015). However, these serologic tests based in antibodies search have some problems regarding the large amount of false negative results, probably due to the "window period" - the time between potential

exposure and an accurate test result – in which IgM antibodies are not produced or are still not detectable. Consequently, molecular approaches would be helpful in testing patients early in the disease, before an antibody response develops, and in patients presenting non classic symptoms.

In the last years, the molecular tests for LD diagnosis are becoming increasingly more sensitive, which is quite important since the quantification of *Borrelia* genospecies in ticks and biological samples has revealed a heterogeneous distribution, ranging from one to more than 1000 *Borrelia* genome equivalents (Stunzner et al., 1998; Rauter et al., 2002). Thus, the development of molecular approaches with higher accuracy, sensitivity and easy to perform is a priority. Isothermal amplification assays such as LAMP gained interested in more recent years, due to its rapidity, sensitivity and easy to perform characteristics. LAMP allows to achieved a high number of DNA copies ($\approx 10^9$) in less than one hour (Notomi, 2000). One of the most important points in LAMP is the design of appropriate primers since at least four primers that recognize six distinct regions on the target DNA are required, being possible to increase the specificity and rapidity of the assay by adding an extra pair of primers, defined as loop primers.

One of the advantages of LAMP is that is does not require the use of sophisticated equipment, being possible to perform the reaction in a water bath or in a thermoblock. Amplification results can be visualized at naked eye or by adding intercalating fluorescent dyes (Mori et al., 2006; Notomi et al., 2015).

Therefore, our aim was to develop and optimize a LAMP assay for the identification of four of the most prevalent *B. burgdorferi* s.l. genospecies in Europe. This assay consisted of two duplex LAMP's (dLAMP) copulated to a Lateral Flow Device, namely to chromatographic strips, that can detect biotin-labelled DNA fragments hybridized with complementary FITC-labelled probes. The molecular target was the *flaB* gene that presents a recognized interspecific variability, allowing to distinguish the genetic heterogeneity between *Borrelia* spp. isolates (Shih and Chao, 2002), being also considered the most sensitive target for the detection of *Borrelia* spirochetes (Wodecka, 2011).

After the primers design for each *Borrelia* genospecies, the sensitivity and specificity was tested, and unfortunately each set of primers was not specific for the respective *B. burgdorferi* s.l. genospecies, since it amplified not only the right genospecies as also one or even the three others genospecies, which happened for *B. lusitaniae* and *B. burgdorferi* s.s. sets. This lack of specificity was a surprise since we assumed that the four to six different nucleotides between each set of primers were enough to distinguish the four genospecies, since these differences were sufficient in the development of a tetraplex real time PCR, for the differentiation of the same four genospecies (data in submission).

A study developed by Mori et al., (2006), showed another approach, using LAMP technique as base, that allowed to differentiate two different virus, by adding a low-molecular weight polymer PEI (Polyethylenimine) positively charged, to LAMP reaction solution, forming an insoluble complex with high molecular weight DNA. By centrifugation, a colorful pellet is obtained, due to the fluorescent dye previously added at the 3' end of the loop primers.

Therefore in order to accomplish our initial aim, and since we could not use our loop primers because they were not species specific, we designed specific probes for each genospecies labelled with different fluorochromes that were complementary to the product obtained by LAMP amplification with *B. lusitaniae* primers sets, since it was one of the sets that allowed to amplify the four genospecies (data not showed). However, after adding PEI, all pellets presented florescence when submitted to UV light, regardless if the sample tested had or not the complementary DNA to the respective probe (data not showed). Therefore, and once again, it was not possible to achieve the desired specificity for each genospecies. These results can be related with the PEI concentration, that if is in excess, PEI will bind to the free probes that were not linked to the respective DNA, yielding to false positives. Due to this reasons we abandoned this approach.

Nevertheless, we chose to proceed with a LAMP assay specific not for each of the four genospecies, but for the *B. burgdorferi* s.l. complex by using the *B. lusitaniae* primers sets. The sensitivity of this set was determined and as expected, it was better for *B. lusitaniae* DNA when compared with the other three genospecies. When compared with the two nested-PCR's, targeting the IGS region, the *flaB* gene, and the real-time PCR, also targeting *flaB*

gene, LAMP assay showed a lower sensitivity, even for *B. lusitaniae*. However, a study performed by Pooja et al., (2014), comparing the sensitivity of LAMP assays for several bacteria, showed sensitivity values of 0.005pg/µl for *Mycobacterium tuberculosis* and 2500pg/µl for *Staphylococcus aureus*, therefore allowing us to verify that our values are not so different from the ones obtained in this study. Several recent studies conclude that LAMP technique has a sensitivity similar to a nested-PCR and 10 times more sensitive than the conventional PCR (Aryan et al., 2010; Zhou et al., 2014).

As far as we know, there are only two other studies concerning the development of LAMP assays to detect the *B. burgdorferi* s.l. complex. One is based on the 16S (*rrs*) gene for tick samples, where the sensitivity was higher than our, being 0.2 pg (10^2 copies/µl) for *B. afzelii* and *B. burgdorferi* s.s., and 0.02 pg (10 copies/µl) for *B. garinii* (Yang et al., 2013); and the other study combined LAMP technique with a nested-PCR for human serum samples (Zhang et al., 2015), where the authors used a Loopamp DNA amplification kit (Eiken Chemical Co. Ltd., Tokyo, Japan) and achieved a sensitivity of 0.02 pg (10 copies//µl) for the four tested genospecies, namely *B. afzelii, B. garinii, B. burgdorferi* s.s. and *B. valaisiana*.

Beyond trying to achieve a better sensitivity with our LAMP assay, during this process we came across with unspecific amplifications in the negative controls that contained water instead of DNA. Several other authors reported the same problem, and explained it by the presence of primers dimers, since there are at least six primers in reaction or eight considering the loop primers, resulting in false positives (Wang et al., 2015b). These same authors also published a study where they compared the results obtained by other authors that used in house LAMP for 12 bacteria species, with the results obtained by using a commercial kit "Isothermal Master Mix, OptiGene, Horsham, UK" (Wang et al., 2015a), where they observed that in all in house LAMP reactions unspecific amplifications in the negative controls were obtained, however, the same was not observed with the commercial kit, proving that this undesired amplifications can be controlled or eliminated by using commercial kits where the Mg^{2+}, DNTPs and DNA polymerase concentrations are more strictly controlled.

Finally the LAMP technique here presented for *B. burgdorferi* s.l. genospecies differentiation, has to be improved, probably by designing new sets of primers in a different molecular target. However, this technique showed to be easy to perform, and rapid, since it was possible to achieve an amplification in 45 min, being the products visualized by naked eye. Although the main objective of this study has not been fully achieved, important steps were taken in order to maximize in a short-term, a technique capable of identifying spirochetes of *B. burgdorferi* s.l. complex at an early stage of the spirochetal infection.

From our experience this technique could be helpful in addressing the lack of diagnostic tools, capable of detecting the spirochetes in an early stage when the bacteria inoculum transmitted by the vector is very low.

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Chapter 6

Concluding remarks and perspectives

6. Concluding remarks and perspectives

Lyme disease is increasing rapidly in many parts of the world and is the most commonly occurring vector-borne disease in Europe and the USA. In Portugal, despite LD has been identified twenty five years ago, this zoonosis still remains underdiagnosed and underreported, being often assigned by our physicians as a pathology only present in USA. Moreover a gold standard test with standardized diagnostic criteria for LD diagnosis, is not yet establish, existing a variety of direct and indirect approaches, most of them used incorrectly and inadequate to the evolution stage of the disease. Thus, the studies developed in the present thesis aimed to contribute to:

- a bio-ecological characterization of the ixodofauna in nine districts across mainland Portugal, where the presence of *I. ricinus* ticks were previously reported, and to determinate *B. burgdorferi* s.l. infection rate in the collected ticks;
- a better knowledge of the eco-epidemiology of *B. burgdorferi* s.l. genospecies and Relapsing Fever *Borrelia* at the vector and animal hosts level;
- a more improved molecular diagnosis of LD, by the development and evaluation of two molecular tools namely a *TaqMan* real-time PCR algorithm and a isothermal amplification protocol for the identification of four of the most prevalent genospecies of *B. burgdorferi* s.l. in Portugal.

In the captures carried out in the nine districts, several tick species were possible to collect from the vegetation (n = 4251) as well as from the hosts (n = 2171). The most widespread tick species was *R. sanguineus* regarding the vegetation and the animal hosts, although we could not cover all Portuguese districts, an possible expansion of tick species into new regions was noticed namely *D. reticulatus* in Braga district and *I. ricinus* in Aveiro district, since there are not records of the presence of these species in the two districts. This fact may have numerous consequences, including modifications in their ecological characteristics, impacts on the dynamic of local host populations, and also important implications when considering the tick-borne pathogens that could affect humans and other animal species. Also

B. burgdorferi s.l. infection rate was firstly determined by two nested-PCR targeting the *flaB* gene and the intergenic space region 5S-23S, being *I. ricinus* nymphs the more infected stage, although, other tick species were also infected by these pathogenic agents.

The positive ticks were sequenced and the tick samples that were not identified as *B. burgdorferi* s.l. species, were subjected to two other PCR protocols targeting the *glpQ* gene, and the 16S rDNA gene. The sequencing results revealed that *B. lusitaniae* was the most prevalent species in *I. ricinus* tick from Vila-Real, Lisboa, Setúbal and Faro districts. Moreover, *B. garinii*, *B. burgdorferi* s.s., *B. valaisiana* and *B. afzelii* DNA were also identified in several ticks species rather than *I. ricinus*, namely *D. marginatus* from Braga district, *R. sanguineus* from Braga, Vila-Real, and Évora districts and *Hy. lusitanicum* and *H. punctata* from Lisboa district. These results confirm previous reports indicating a countrywide distribution of *B. burgdorferi* s.l. genospecies in questing ticks, being *B. lusitaniae* the most prevalent species at the vector level.

Unexpectedly, *B. miyamotoi* DNA was identified for the first time in the country, in a questing *I. ricinus* nymph from Lisboa district, although no human cases have been identified so far in the country, this species has been associated to human disease in others countries from Europe. Despite this species belongs to Relapsing Fever *Borrelia* group, whose spirochetes are usually transmitted by soft-body ticks, *B. miyamotoi* has been found in hard-body ticks from America, Europe and Asia continents, mainly in *Ixodes* genus, revealing an extensive geographic distribution. Therefore, further studies involving more collections in other districts are needed, to better understand the possible spread of *B. miyamotoi* in Portugal, contributing to the determination of the human risk of exposure to the vector and the bacteria.

Additionally, DNA from two possible new Relapsing Fever like *Borrelia* species were also identified, one in five pools of questing larvae and one questing nymph of *H. punctata* tick from Lisboa district, and the other in two questing *R. sanguineus* females from Braga and Évora districts. By phylogenetic analysis of 16S rRNA, *flaB* and *glpQ* sequences, these two novel species form two independent clusters placed in a larger subgroup of Relapsing Fever *Borrelia* that included *B. theileri*, *B. lonestari* and a number of unclassified spirochetes. Due

to the small number of positive samples it is not clear if these bacteria are restricted to tick species or to the area where they have been found, therefore isolation of these spirochetes *in vitro*, their characterization and the role in human and veterinary disease, will be the focus in a future research, associated to a more widespread collection of ticks across the country.

Concerning the ticks collected from the hosts, it was possible to identify DNA from several pathogens, namely *Anaplasma* spp., *Babesia* spp., *B. burgdorferi* s.l., *Cercopithifilaria* spp., *Hepatozoon* spp., and *Ricketsia* spp., in ticks collected from dogs and cats that belonged to Guarda, Lisboa, Setúbal and Faro districts. *Ri. massiliae* DNA was amplified for the first time in ticks collected from cats, although there is no evidence that it can cause illness or that the animal can play a role in the transmission of this bacterium to humans. Also, *Cercopithifilaria* spp. was detected for the first time in a *R. sanguineus* collected from a dog. Regarding DNA from *B. burgdorferi* s.l., it was possible to identify it in only one tick sample from *R. sanguineus* species collected from a dog in Setúbal district. This study was the first in the country that revealed the presence of protozoa and nematodes with veterinary medical importance, in ticks collected from domestic animals, suggesting a risk of emergence of these tick-borne diseases in domestic animals and in humans. Consequently, more studies on these and other tick-borne agents should be performed in more districts across the country, to better understand its epidemiological and clinical importance.

The studies regarding the research of several tick-borne pathogens in biological samples collected from several wildlife hosts, revealed for the first time the presence of *Anaplasma* spp. and *Theileria* spp. DNA, among red deer, fallow deer and wild boars in central/southern Portugal, however the ability of *Anaplasma platys*, the species identified in red deer and wild boars, to cause disease in these animals has not been established yet. Also, *B. afzelii* DNA, was identified by the first time in serum samples from wild boars from Trás-os-Montes region, indicating that the wild boar hunting dogs may act as a link between the wild and the domestic *Borrelia* transmission cycle, by carrying ticks into the hunter's households, exposing them to a higher risk of tick-borne diseases.

These findings point to the importance of wildlife hosts in maintaining several tick-borne pathogens, representing a risk to veterinary and human health, since the interaction of

different pathogens within the vertebrate host might lead to increased susceptibility to other infections, or to modifications of the pathogenesis of each agent, resulting in high risks to wildlife and human health. Therefore, further epidemiological studies concerning *Borrelia*, *Anaplasma* and *Theileria* species, infecting wild ungulates, are required for a proper infection risk assessment across the country. This approach is important to develop future strategies of prevention and disease control rooted in a multidisciplinary approach that encompasses both human and animal health.

For biological samples collected from domestic animals such as dogs and cats from the southern region of Portugal, DNA from several feline and canine vector-borne diseases agents were identified. Feline samples were positive for *Leishmania* spp., *Hepatozoon* spp., *Babesia* spp., *Anaplasma* spp./*Ehrlichia* spp., *Bartonella* spp., and *B. burgdorferi* s.l., while the canine samples were positive for *Anaplasma* spp./*Ehrlichia* spp., *B. burgdorferi* s.l., *Hepatozoon* spp, and *Leishmania infantum*. The identification of feline and canine vector-borne diseases agents in domestic animals from southern Portugal, some of them of zoonotic concern, reinforces the importance to alert the veterinary authorities for the risk of transmission of these vector-borne agents to other vertebrate hosts, including humans. The use of ectoparasiticides against arthropods and education and awareness of the population must be done, to prevent and avoid the dissemination of these pathogens to other hosts.

Regarding the studies concerning the development and optimization of two molecular techniques for the identification of four of the most prevalent genospecies of *B. burgdorferi* s.l. in Portugal, the two-step multiplex *TaqMan* real-time PCR assay targeting the *flaB* locus, proved to be an efficient method when screening for *Borrelia* infection in tick samples, and a promising tool for early diagnostic purposes on clinical samples. Moreover, the ability to detect four of the most prevalent *B. burgdorferi* s.l. genospecies in Europe, in a single-run is a time-saving factor, besides the cost reduction when compared with the conventional PCR and sequencing methods. Concerning the LAMP technique some problems occurred during its optimization, since the designed primers were not specific for each *B. burgdorferi* s.l. genospecies, but only for the complex, and also the presence of unspecific amplifications at the negative controls level. For a better result, this technique will be enhanced in the future,

by designing new sets of primers for each *B. burgdorferi* s.l. genospecies, using a different molecular target, to improve its sensitivity in order to allow the detection of low bacteria inoculum at the vector level, and also in biological samples from the vertebrate hosts, including humans.

Finally, and after the achievement of the proposed objectives in this thesis, concerning *Borrelia* eco-epidemiology at the ticks and hosts level, and the development of diagnostic approaches for a better molecular diagnosis of Lyme disease, it is imperative to think ahead and point to the new challenges open now. Thus, in terms of perspectives, we highlight some lines for 'potential' future research:

i) pursue the study of the Relapsing fever *Borrelia* species now identified, including *B. miyamotoi*, besides the two 'new' species, through a study involving the whole country;

ii) apply analytical techniques and data simulation methods to know spatial patterns, quantify trends and assess the *Borrelia* infections in humans as well as in vectors and reservoir hosts, in order to increase the understanding of the epidemiology, risks and impact of Lyme borreliosis in Portugal, contributing to Lyme European network;

iii) improve the diagnosis tools, namely the LAMP technique to be applied in laboratories with low resources and limited equipment.

Several factors have contributed to the emergence of LD and other tick-borne diseases in Europe. Namely, the expansion of the outdoors activities and travels among Europeans, resulting in an increased contact with ticks and an higher risk of contracting tick-borne diseases; the development of new techniques for the detection of the bacteria; the use of molecular methods for their characterization and the need to answer the questions of clinicians, in the presence of new or unusual cases. In addition, it is important to keep in mind that climatic change is another factor, which could modify the epidemiology of both human and animal tick-borne diseases, contributing to the emergence of new diseases in the future.

Therefore, based on these assumptions, it is assumed that the work presented in this thesis can contribute for a better knowledge of the bio-ecology of ixodids in the selected districts,

and for *Borrelia* eco-epidemiology, where two possible new Relapsing Fever like-*Borrelia* species were identified in hard-body ticks, indeed a new and surprising finding.

Moreover, after more than 30 years of *Borrelia* spirochete isolation, LD diagnosis still remains a laboratorial problem, since the methodologies are not standardized worldwide. This fact is probably related to the existence of several species belonging to *B. burgdorferi* s.l. complex, each one presenting different tropisms, resulting in diverse clinical pictures. Thus, in this thesis two molecular tools were developed for a more accurate and prompt diagnosis of LD, one of which allowing the identification of *B. burgdorferi* s.l. genospecies involved in the infection, being of great help to clinicians, allowing them a more effective and targeted treatment.