Dissemination of *Borrelia burgdorferi*, the Agent of Lyme Borreliosis, in Comparison to *Borrelia persica*, the Agent of Tick-Borne Relapsing Fever, in a Murine Model

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For my family

For Stefan Gruhler and his family

Content

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Abbreviations

%	percent
°C	grad Celsius
µg	microgram
µl	microliter
µm	micrometer
ACA	acrodermatitis chronica atrophicans
approx.	approximately
B.	*Borrelia*
Bb	*Borrelia burgdorferi*
BBB	blood-brain-barrier
*Bb*sl	*Borrelia burgdorferi* sensu lato
*Bb*ss	*Borrelia burgdorferi* sensu stricto
BCA	bicinchoninic acid
bp	base pair(s)
Bp	*Borrelia persica*
BSK-H	Barbour-Stoenner-Kelly H
CbiA	complement binding and inhibitory protein A
CDC	Centers for Disease Control and Prevention
cm	centimeter
cm^2	square centimeter
CNS	central nervous system
CRASPs	complement regulator-acquiring surface proteins
CSF	cerebrospinal fluid
Ct	threshold cycles
DbpA/B	decorin-binding protein A/B
Dbps	decorin-binding proteins
DCs	dendritic cells
DNA	deoxyribonucleic acid
dsDNA	double strand DNA
ECM	erythema chronicum migrans
e.g.	for example
ELISA	enzyme-linked immunosorbent assay

EM	erythema migrans
Erp	OspE/F-related proteins
et al.	and others
FH	factor H
FHBPs	factor H-binding proteins
flaB	flagellin B
flaB	flagellin B gene
g	gram
GlpQ	glycerophosphodiester-phosphodiesterase
GlpQ	glycerophosphodiester-phosphodiesterase gene
GV-SOLAS	Gesellschaft für Versuchstierkunde/Society of Laboratory Animal Science
h	hour
hrs	hours
HTBRF	hard tick-borne relapsing fever
I.	*Ixodes*
ID	intradermal(ly)
i.e.	that is to say
IFA	immunofluorescent antibody analysis
IFNs	type 1 interferons
Ig	immunoglobulin
IP	intraperitoneal(ly)
Irac	*Ixodes ricinus* anticomplement
Isac	*Ixodes scapularis* anticomplement
IV	intravenous(ly)
IVC	individually ventilated cage
kDa	kilo-Dalton
KELA	kinetic ELISA
kg	kilogram
LA	Lyme arthritis
LB	Lyme borreliosis
LBRF	louse-borne relapsing fever
LC	Lyme carditis
LD	Lyme disease
LIA	line immunoblot assay

LMU	Ludwig-Maximilians-Universität
LNB	Lyme neuroborreliosis
log	logarithm
lp	linear plasmid
LPS	lipopolysaccharide
Mb	Mega base pairs
mg	milligram
min	minute
ml	milliliter
MLST	multilocus sequence typing
NCBI	National Center for Biotechnology Information
NK	natural kill
NLRs	NOD-like receptors
nM	nanomolar
no.	number
NOD	nucleotide-binding oligomerization domain
NOD-SCID	non obese diabetic-severe combined immunodeficiency
nov.	novel
NTC	no template control
O.	*Ornithodoros*
OmCI	*O. moubata* complement inhibitor
ospA	outer surface protein A gene
OspA-F	outer surface protein A-F
Osps	outer surface proteins
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
p.i.	post inoculation
PRRs	pattern recognition receptors
qPCR	quantitative real-time PCR
RF	relapsing fever
rRNA	ribosomal ribonucleic acid
rrs	16S rRNA gene
s	second
Salp	salivary protein

SB	subcutaneous(ly)
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPF	specific-pathogen-free
spp.	species (plural)
STARI	southern tick-associated rash illness
STBRF	soft tick-borne relapsing fever
TBDs	tick-borne diseases
TBRF	tick-borne relapsing fever
TLRs	Toll-like receptors
TOT	transovarial transmission
TTI	transfusion-transmitted infections
UK	United Kingdom
USA	United States of America
var.	varietas
Vlps	variable large proteins
Vls	variable major protein-like sequence
vls	variable major protein-like sequence gene
VlsE	variable major protein-like sequence expression
vlsE	variable major protein-like sequence expression gene
Vmps	variable-major-proteins
Vsps	variable small proteins
Vtp	variable tick protein
WHO	World Health Organization
yrs	years

I INTRODUCTION

Various species in the genus *Borrelia* (*B.*) are the etiological agents of tick-borne relapsing fever (TBRF) and Lyme borreliosis (LB) infections in vertebrate hosts including humans (SCHWAN & PIESMAN, 2002). *B. persica* (*Bp*), a recently successfully in vitro culturable TBRF spirochete (ZAMANI et al., 2014), is transmitted by the argasid soft (fast-feeding) tick *Ornithodoros* (*O.*) *tholozani*. This bacterium represents the most significant and prevalent species causing human TBRF in the Central Asian and Middle Eastern countries (ASSOUS & WILAMOWSKI, 2009; OSHAGHI et al., 2011). Contrariwise, species of the *B. burgdorferi* sensu lato (*Bb*sl) complex are vectored by *Ixodes* (*I.*) hard ticks and mainly cause LB, namely, Lyme disease (LD). Because of the frequent association with human infections, the most important genospecies of *Bb*sl include: *B. burgdorferi* sensu stricto (*Bb*ss) in both North America and Europe, *B. afzelii*, *B. garinii*, and *B. bavariensis* in Europe and Asia (RIZZOLI et al., 2011; BORCHERS et al., 2015).

Consistent with other well identified TBRF species, e.g., *B. hermsii* and *B. turicatae* that are primarily endemic in the United States (DWORKIN et al., 2002), *Bp* has also been detected in large quantities in the peripheral blood of infected animals (RAFINEJAD et al., 2011; BANETH et al., 2016). In addition, blood samples from human patients with history of fever attacks showed numerous *Bp* spirochetes (DE VERDIÈRE et al., 2011; KUTSUNA et al., 2013). During or after the appearance of spirochetemia in the bloodstream, borrelial organisms can be found in the brain tissues of infected mice (ADDAMIANO & BABUDIERI, 1957; SCHWARZER et al., 2016). However, still no information is available to clearly characterize the dissemination pathway of *Bp* in experimental animals.

In comparison to *Bp*, LB spirochetes are deposited slowly during the *Ixodes* tick bite. At early days of infection (2 - 4 weeks), cutaneous inflammatory responses (erythema migrans, EM) around the bite site are developed frequently (STEERE, 1989; SCHWAN & PIESMAN, 2002). Clinically, *Bb*sl cells have been examined in this early stage of infection by means of PCR or bacteriological culture from large volumes of blood or serum samples from the Lyme disease patients (WORMSER et al., 1998). Without treatment or being treated insufficiently, *Bb*sl infections often result in multisystemic infectious disease such as Lyme carditis (LC), Lyme arthritis (LA), or acrodermatitis chronica atrophicans (ACA) (STEERE et al., 1987; STEERE et al., 2016). It is generally believed that mechanisms of these manifestations are

reliant on the route of borrelia dissemination. However, the exact dissemination pathway of these bacteria in vivo is still in debate and not evidently understood so far. In response to these questions, some authors hypothesized that LB borreliae employ a hematogenous route, similar to some identified TBRF *Borrelia* spp. such as *B. hermsii* and *B. turicatae*, which firstly enter vasculature near the site of the tick bite and subsequently exit from the vasculature to various tissues (RISTOW et al., 2015; HYDE, 2017). However, some studies suggested the progress of chronic LB is not due to transmission of *Borrelia* via the bloodstream, but instead due to the migration of spirochetes through various tissues (STRAUBINGER et al., 1997).

Do TBRF and LB species, e.g., *Bp* and *Bb*ss, have the same hematogenous dissemination route, or not? It is of scientific importance to define the exact dissemination pathways of the two biologically different pathogens in the mammalian host. In the present study, we developed a novel long-term murine model to examine the dissemination route of host-adapted *Bp* and *Bb*ss organisms in vivo post intradermal (ID) and strict intravenous (IV) inoculation in immunocompetent mice. The objectives of this investigation were to (1) provide an animal model with a precise and defined infection route; (2) investigate the population dynamics of borrelia organisms disseminated in the bloodstream of the immunodeficient and immunocompetent mice; (3) study whether *Bp* and *Bb*ss disseminate into tissues of mice after ID or IV inoculation; (4) characterize the immune response against host-adapted borrelia organisms in immunocompetent mice.

Based on the data obtained, it is concluded that our long-term murine infection model was successfully established. This newly established murine model is a reliable tool to shed more light on the dissemination route (via blood *versus* via tissue) of *Bp* and *Bb*ss organisms in immunocompetent mice.

II LITERATURE REVIEW

1 Historical background

1.1 Tick-borne relapsing fever (TBRF)

Tick-borne relapsing fever (TBRF) caused by the spirochete *Borrelia* (*B.*) *duttonii* was first described in humans in East Africa in 1905 with a feature of acute fever episodes separated by afebrile intervals (DUTTON et al., 1905; BURGDORFER, 2001). In many states of the USA, human cases of TBRF were reported during the first half of the 20th century (DWORKIN et al., 2002). In Persian of Iran, the first clinical description of TBRF, which was spread to humans by the soft tick *Ornithodoros* (*O.*) *tholozani*, was published as early as in 1882 (THÉODORIDÈS, 1998). Its causative agent, *Spirochaeta persica*, was first isolated from the blood of a patient in 1913 (DSCHUNKOWSKY, 1913; EUZÉBY, 1997). In the following decades, *B. persica* (*Spirochaeta persica*, *Bp*) vectored by *O. tholozani* was clearly identified as the agent causing TBRF (ADLER et al., 1937; BABUDIERI, 1957; SKERMAN et al., 1980). Nowadays, *Bp* is known to be the main cause of TBRF in Central Asia and Middle East areas (OSHAGHI et al., 2011).

1.2 Lyme borreliosis (LB)

Lyme disease (LD) was originally named as Lyme arthritis (LA) in 1975 after the town of Lyme in Connecticut, USA, where a bizarre cluster of arthritis cases was reported from adolescents (STEERE et al., 1977). A skin lesion erythema chronicum migrans (ECM) was a typical manifestation in the early phase of LD (STEERE & MALAWISTA, 1979). In 1982, the etiological agent of LD was first isolated from the midgut tissues of the hard tick *Ixodes* (*I.*) *scapularis* and was named *B. burgdorferi* (*Bb*) in honor of its original discoverer (BURGDORFER et al., 1982; JOHNSON et al., 1984). In Europe, individual ECM had first been documented in 1909 (AFZELIUS, 1910) and the tick species *I. ricinus* was identified as a vector related to this clinical sign (GELBJERG-HANSEN, 1945; THÖNE, 1968). After the 1980s, the species *B. burgdorferi*, *B. afzelii* and *B. garinii* were further determined as etiological agents to cause LD in Europe and Asia (BORCHERS et al., 2015). Until today, Lyme disease or, more precise, Lyme borreliosis (LB), is considered one of the most prevalent tick-borne diseases (TBDs) in Europe (VAN DEN WIJNGAARD et al., 2017) and in the United States (PENG et al., 2017).

2 Etiology and epidemiology

2.1 Taxonomy and nomenclature of *Borrelia* spirochetes

Spirochetes in the genus *Borrelia* belong to the family Spirochaetaceae (BURGDORFER et al., 1982). In addition to other three families within the order Spirochaetales, Spirochaetaceae belongs to the class of Spirochaetes, which is incorporated in the Phylum of Spirochaetes (EUZÉBY, 1997). According to genomic, genetic and phylogenetic studies on nucleotide and protein signatures (ADEOLU & GUPTA, 2014; OREN & GARRITY, 2016), *Borreliella* ("borrelia-like") gen. nov. was proposed to differentiate the pathogens that cause LB from those that cause relapsing fever (RF; maintaining the genus *Borrelia* affiliation). However, the splitting of genus *Borrelia* into two taxonomical genera groups has not been accepted due to some criticism and inadequate evidence (MARGOS et al., 2017). Nevertheless, 42 *Borrelia* species have nowadays been recognized and divided into two groups responsible for RF and LB (EUZÉBY, 2012). Except for *B. recurrentis*, which is transmitted by the body louse *Pediculus humanus* and causes epidemic louse-borne relapsing fever (LBRF) in humans, all other known RF-related *Borrelia* species are tick vectored and therefore named tick-borne relapsing fever (TBRF) (Figure 1) (BARBOUR & HAYES, 1986; MARGOS et al., 2017). Generally, TBRF and LB *Borrelia* spirochetes are transmitted by soft ticks of the genus *Ornithodoros* and hard ticks of the genus *Ixodes*, respectively. However, a TBRF inducing species, *B. miyamotoi,* shares the same *Ixodes* vector as LB species (TAKANO et al., 2014). Thus, TBRF can be divided into soft tick-borne relapsing fever (STBRF) and hard tick-borne relapsing fever (HTBRF) (Figure 1) (TALAGRAND-REBOUL et al., 2018).

The classification of *Borrelia* is as follows:

Order: Spirochaetales

Family: Brachyspiraceae

Family: Brevinemataceae

Family: Leptospiraceae

Family: Spirochaetaceae

Genus: *Borrelia*

Species: *Borrelia* spp. associated with LB

Borrelia spp. associated with RF (louse- and tick-borne)

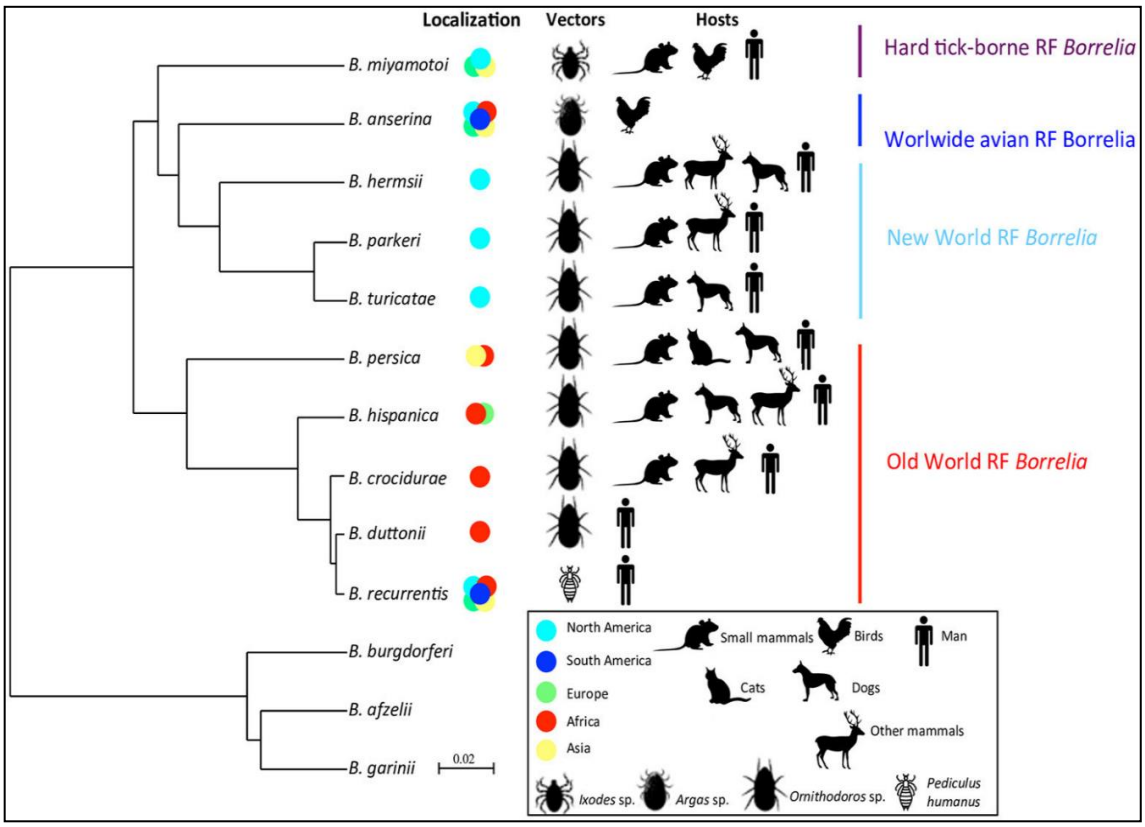

Figure 1: Phylodendrogram of average nucleotide identity values among relapsing fever borrelia genomes

LB borrelia genomes are shown as an outgroup.
Cited from the reference (TALAGRAND-REBOUL et al., 2018)

Among the *Borrelia* species *Bp* is one of the most important and prevalent pathogens of TBRF in humans (ASSOUS & WILAMOWSKI, 2009). Based on the flagellin B gene (*flaB*) and 16S ribosomal ribonucleic acid (rRNA) gene (*rrs*) sequencing of *Bp* isolated from *O. tholozani* ticks and TBRF human blood samples, a separate cluster has been formed from the other African (Old World) RF *Borrelia* in the phylogenetic tree (Figure 1) (ASSOUS et al., 2006; SAFDIE et al., 2010; TALAGRAND-REBOUL et al., 2018). On the other hand, various LB species have been classified in the *B. burgdorferi* sensu lato (*Bb*sl) complex and divided into 21 validated genotypes on the basis of deoxyribonucleic acid (DNA) relatedness (LOHR et al., 2018). Of them, the closely related species that induce most human LB cases have been classified as distinct genospecies such as *B. burgdorferi* sensu stricto (*Bb*ss), *B. afzelii*, *B. garinii* (Figure 1) by DNA-DNA hybridization and 16S rRNA sequencing (JOHNSON et al., 1984; BARANTON et al., 1992). Also, multilocus sequence typing (MLST) which conducts targeted gene amplification and sequence analysis of several defined housekeeping genes has been used to distinguish both *Bp* and *Bb*sl species

(MARGOS et al., 2011; SCHWARZER et al., 2015). For example, strains of *B. garinii* OspA[1] serotype 4 (a rodent-associated ecotype) have been newly designated *B. bavariensis* species by MLST in humans and ticks in Europe and Asia (MARGOS et al., 2013). In particular, lack of the glycerophosphodiester-phosphodiesterase gene (*GlpQ*) differentiates LB from TBRF *Borrelia* spp. that possess this gene (PETTERSSON et al., 2007).

2.2 Pathogenic TBRF and LB *Borrelia* species

2.2.1 TBRF *Borrelia* species throughout the world

TBRF is an important global infection disease that is caused by several *Borrelia* species (Figure 1). The *Ornithodoros* spp. ticks, the transmitting vectors of TBRF, are present worldwide and are closely associated with their animal hosts (Figure 1). Naturally occurring infections with TBRF spirochetes have been observed in a diversity of mammals including squirrel monkeys, opossums, and armadillos, calves, horses and humans (LOPEZ et al., 2016). However, the impact of TBRF on the health of domestic and wild animals is mostly understudied (SCHWAN et al., 2005). Nevertheless, only few cases of infection in dogs, cats, domestic pigs and horses from some limited parts in the world have been reported with veterinary importance. In comparison, clinical TBRF cases in humans have been clearly documented in most areas of the world and remain a noticeable public health concern (ELELU, 2018).

Among pathogenic TBRF spp., *B. hermsii* and *B. turicatae* are the primary entities in the United States. Associated with the geographical distribution of their transmission vectors, *O. hermsii* and *O. turicata*, respectively, this disease is endemic in moderate to high-elevation and coniferous forests of the United States (DWORKIN et al., 2002). *B. parkeri,* another TBRF spirochete that was recovered from *O. parkeri*, shares similar geographic distribution to *B. hermsii* and can also pose risks to both animals and humans (THOMPSON et al., 1969; BARBOUR & CAMPEAU MILLER, 2014). Of the reported TBRF cases (n=504) in humans from 1990 to 2011 in the USA, most are caused by *B. hermsii* and approx. 70% cluster in California, Washington, and Colorado (FORRESTER et al., 2015). In Africa, the main circulating species are *B. crocidurae* in Western and Northern Africa and *B. duttonii* in Eastern, Central and Southern Africa (VIAL et al., 2006; TRAPE et al., 2013). *B. hispanica* is found in some Mediterranean countries such as Spain, Portugal, Cyprus,

[1] OspA: outer surface protein A

Greece, and North Africa (REBAUDET & PAROLA, 2006; VIAL et al., 2006; TRAPE et al., 2013). In certain areas of Asia, Europe and USA, *B. miyamotoi* has been reported (TALAGRAND-REBOUL et al., 2018).

Through the Central Asia (Kazakhstan, Kyrgyzstan, Tajikistan, Turkmenistan, Uzbekistan) and Middle East (Iran, Iraq, Syria, Jordan, Turkey, Israel, Egypt) region, clinical human cases of TBRF are confirmed and associated with *Bp* (PAROLA & RAOULT, 2001; ASSOUS et al., 2006). Especially in Israel, Iran and Jordan, *Bp* infection in persons have been detected frequently (DE VERDIERE et al., 2011). Although other species of *B. caucasica*, *B. latyschewii*, *B. microti* and *B. baltazardi* have been described, they are not prevalent in these areas (KARIMI et al., 1979; GOUBAU, 1984; AGHIGHI et al., 2007). In some epidemiological studies in Iran, cases of TBRF caused by *Bp* were mostly found in rural areas, especially correlated with young people (e.g., students and children) (ARSHI et al., 2002; ASL et al., 2009; RAFINEJAD et al., 2012; KASSIRI et al., 2014). Provinces of Ardabil, Hamadan, Zanjan and Kurdistan are highly endemic regions (MASOUMI ASL et al., 2009). In Israel and Jordan, *Bp* infection is often found in hikers who enter the tick infested habitats but normally without necessary precautions. In summer and autumn during the year, TBRF occurs frequently, because of human outdoor activities exposed to tick vectors (ASSOUS & WILAMOWSKI, 2009; KASSIRI et al., 2014). Especially in Israel, 30% - 60% of caves were found to be infested by ticks of *O. tholozani*. Therefore, TBRF in this country has traditionally been called cave fever (SIDI et al., 2005).

2.2.2 LB *Borrelia* genospecies in the Northern Hemisphere

Of the Lyme *Borrelia* spp., *Bb*ss (also referred to *Bb*) predominates in the United States and less extensively in Europe, whereas *B. afzelii* and *B. garinii* are more endemic than *B. spielmanii* in Europe; *B. bavariensis* is widely distributed in Europe and Asia. All of these five genospecies are particularly human pathogenic agents of LB (Table 1) (MARGOS et al., 2013; SCHOTTHOEFER & FROST, 2015; STEERE et al., 2016). Additionally, *Bb*ss species in the USA has been solely detected to be pathogenic in dogs, whereas only DNA of *B. afzelii* and *B. garinii* was found in naturally infected dogs in Europe and Asia (HOVIUS et al., 1999; SPECK et al., 2001). The other species, such as *B. lusitaniae*, are only occasionally associated with human disease while the pathogenicity of other tick-isolated species (e.g., *B. valaisiana*, *B. americana*, *B. californiensis*, and *B. caroliniensis*) in persons

or animals has not been demonstrated so far (COLLARES-PEREIRA et al., 2004; DIZA et al., 2004; KRUPKA & STRAUBINGER, 2010).

In humans, different genospecies seem to be variably associated with the particular clinical manifestations in some organs (skin, nervous system, joint) of the LB patients, although they share some common clinical signs such as EM and an influenza-like illness. In Europe, the most common disseminated symptom is Lyme neuroborreliosis (LNB) attributed to *B. garinii* (mostly) and *B. bavariensis*. *B. afzelii* is principally involved in cutaneous manifestations such as EM and ACA. *Bb*ss, however, is most frequently the etiological agent for LA of patients (STANEK et al., 2012; VEINOVIĆ et al., 2013; COIPAN et al., 2016). The heterogeneity of the *Bb*sl strains, which are transmitted by different species of *Ixodes* ticks, is possibly the main factor accounting for the variances in the clinical symptoms of human LB from/in different geographical regions (MARGOS et al., 2011). A study involving at least 26 European countries revealed that *Bb*sl infection occurred from Italy to Iceland and from Portugal to Russia. The proportion of the population showing positive for *B. burgdorferi* antibodies differs in various countries (approx. 5 - 25%) (HABÁLEK & HALOUZKA, 1997). There is no obvious sex bias of LB while age distribution is generally bimodal, with the highest occurrence rates seen in children 5 - 9 years of age and in adults aged over 50 years in both the USA and Europe (BORCHERS et al., 2015). Annually, more than 85,000 cases are widespread in Europe, typically in Central (Germany, Austria and Switzerland) and Eastern Europe (LINDGREN et al., 2006). During 2005 - 2010, averagely 106.6 LB cases per 100,000 individuals per year in the USA have been reported by the Centers for Disease Control and Prevention (CDC) (NELSON et al., 2015). However, recent modeling investigations based on claims data suggest significant under-reporting and predict much higher quantities of LB cases annually in the USA (>300,000) and Germany (>200,000) (MULLER et al., 2012; NELSON et al., 2015).

As one of the significant infectious TBDs in the Northern Hemisphere, LB has been described in more than 80 countries (KUGELER et al., 2015; STEERE et al., 2016). Globally, it seems to be on the rise because of climate changes, land use as well as recreational behavior of humans which impact the ticks and thus disease prevalence (LINDGREN et al., 2006). For example, the number of confirmed cases of LB increased approx. eight times between 2004 and 2012 in Canada (OGDEN et al., 2014) and from 27,444 (2007) to 29,513 (2017) in the USA (CDC, 2017).

Table 1: Members of *Bbs*l complex of confirmed or possible human pathogenic significance of Lyme borreliosis

causative agents	typical vectors	geographical distribution	main reservoirs	pathogenicity for humans	clinical signs[a]
Bbss	*I. scapularis*, *I. pacificus*, *I. ricinus*, *I. persulcatus* (?)	North America, Europe	mammals, birds	+ + +	time of onset of clinical signs after exposure: early stage generally 3 - 30 days; influenza-like (e.g., mild fever, malaise, myalgia/arthralgia; *Bbss*); erythema migrans (*Bbss*, *B. afzelii*) time of onset of clinical signs after exposure: late stage generally > 30 days; arthritis; acrodermatitis chronica atrophicans (*B. afzelii*); neurological (Lyme neuroborreliosis, e.g., numbness, Bell's palsy, stiffness of neck, declining memory, sleep disorders; *Bbss*, *B. bavariensis*)
B. afzelii	*I. ricinus*, *I. persulcatus*	Europe, Asia	small mammals	+ + +	
B. garinii	*I. ricinus*, *I. persulcatus*	Europe, Asia	birds	+ + +	
B. bavariensis	*I. ricinus*, *I. persulcatus*	Europe, Asia	small mammals, birds	+ + +	
B. spielmanii	*I. ricinus*, *I. persulcatus*	Europe	garden dormouse	+ + +	
B. mayonii	*I. scapularis*, *I. pacificus*	North America	mammals	+ +	
B. lusitaniae	*I. ricinus*	Europe	lizards	(+)	
B. bissettiae	*I. pacificus*, *I. spinipalpis*, *I. ricinus*	Europe, North America	*Neotoma fuscipes* (wood rat)	(+)	
B. valaisiana	*I. ricinus*, *I. granulatus*, *I. columnae*	Europe, Japan, Taiwan (China), Korea	birds	?	

[a] Clinical signs of LB are based on the confirmed human pathogens of *Bbss*, *B. afzelii*, *B. garinii*, *B. bavariensis*, and *B. spielmanii*.
Data modified from references (STONE & BRISSETTE, 2017; LOHR et al., 2018)

2.2.3 Cell morphology and outer membrane proteins

Spirochetes in the genus *Borrelia* are gram-negative, regularly wavy, slenderly helical, flagellated bacteria (Figure 2). Their length and diameter may range from 8 - 30 µm and from 0.2 – 0.5 µm, respectively. They share the morphological properties but differ within the number of periplasmic flagella and the number and regularity (length, diameter, uniformity and tightness) of the spiral coils (BARBOUR & HAYES, 1986). For example, the number of flagella inserted between inner and outer membranes of the cell is 15 - 30 for RF spirochete (25 - 30 for *Bp*) and only 7 - 11 for *Bb*sl isolate (KARIMI et al., 1979; CHARON et al., 2012). The cytoplasmic membrane of *Bp* is followed by an outer membrane in which the lipoproteins were anchored with their lipid content (BARBOUR & RESTREPO, 2000). In comparison to other TBRF borrelia (e.g., *B. hermsii*), which have been featured of spontaneous antigenic variations on their outer membrane proteins (variable-major-proteins, Vmps) (BARBOUR et al., 1982), *Bp* has been poorly understood in this item. On the other hand, LB spirochetes are irregularly coiled, bound by an inner cytoplasmic membrane and an outer membrane (Figure 2) (BARBOUR & HAYES, 1986; BORCHERS et al., 2015). Particularly, the outer membrane does not contain lipopolysaccharide (LPS) but instead is covered by several outer surface proteins (Osps) (STÜBS et al., 2009). To date, six Osps from OspA to OspF and various other diagnostically-relevant immunodominant protein components of *Bb*sl have been described. Of them, OspA, OspB, and OspC are most significant because their expression is changed to adapt to and to survive in different arthropod and mammalian milieus (DE SILVA & FIKRIG, 1997). The variable major protein-like sequence expression (VlsE) in mammals has been described and characterized with a special serodiagnostic relevance (INDEST et al., 2001; EICKEN et al., 2002). Moreover, LB spirochete harbors a small but unique linear chromosome and a variety of linear (12) and circular (9) plasmids in which relevant Osps are encoded (BRISSON et al., 2012). In 1997, the complete genome size of type strain *Bb*ss B31 was first sequenced with 1,521,419 base pairs (bp) (FRASER et al., 1997), whereas that of *Bp* with 1,784,979 bp (1.7 Mb) was first published in 2014 partly due to the difficulty of in vitro cultivation of this bacteria (ELBIR et al., 2014).

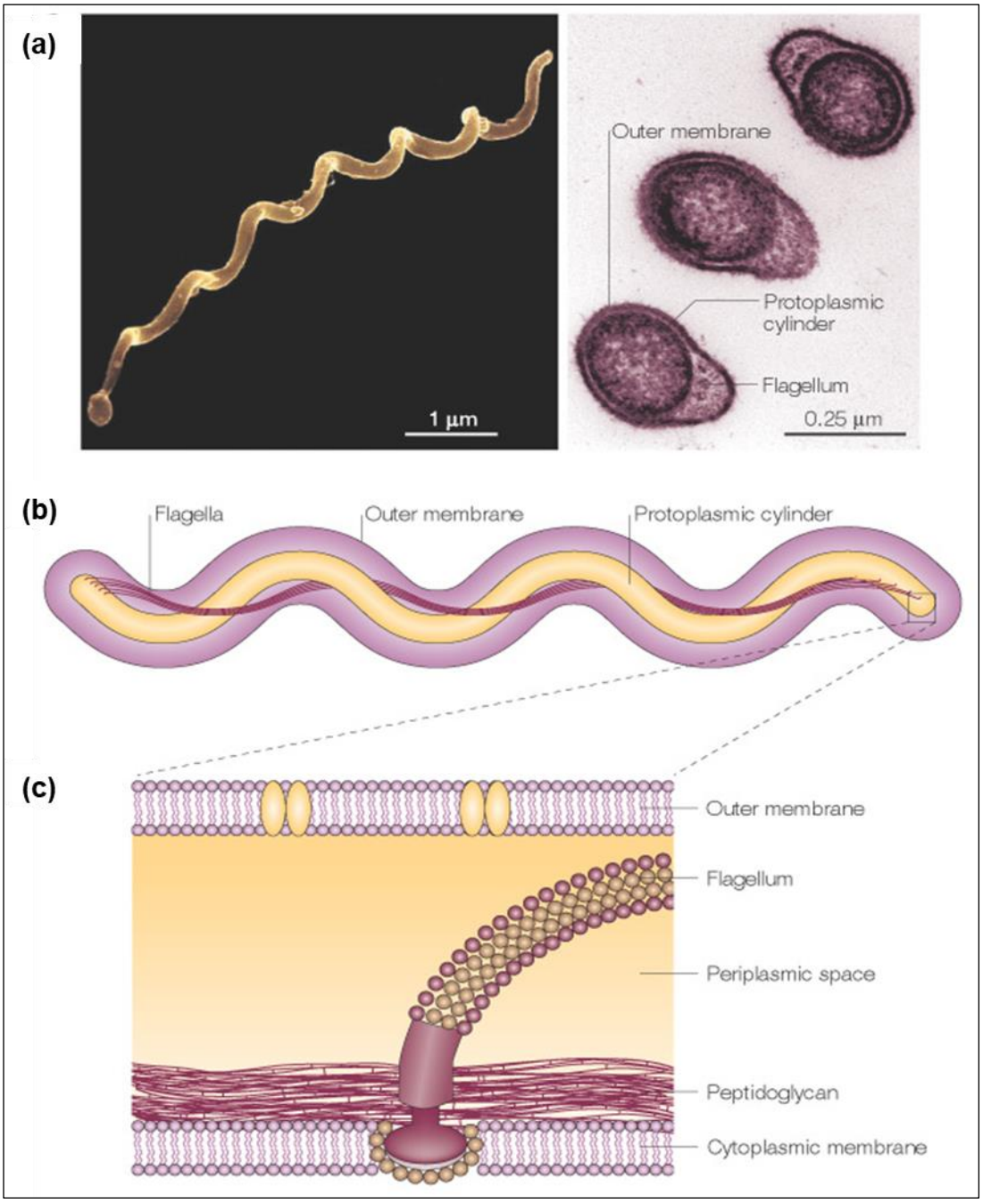

Figure 2: Structure and morphology of *B. burgdorferi*

(a) Scanning (left) and transmission (right) electron micrographs of *B. burgdorferi*. The helical shape of *Borrelia* (left) is imparted by the periplasmic flagella, which can be seen in the cross-sectional view of the spirochete in the transmission electron micrograph. **(b)** Diagram of the spirochete. Flagellar insertion points are located near the termini of the spirochete. Bundles of flagella wind around the flexible, rod-shaped protoplasmic cylinder of *Borrelia* and overlap in the middle. The outer membrane constrains the flagellar bundles within the periplasm. **(c)** Detailed diagram of flagella. Each flagellum is inserted into the cytoplasmic membrane and extends through the cell wall into the periplasm. Flagella are multi-component, complex structures. Spirochetal motility results from coordinated rotation of the flagella.
Cited from Rosa et al. (ROSA et al., 2005)

2.3 The enzootic cycle of TBRF and LB *Borrelia*

2.3.1 Transmission vectors

Ticks are the transmission vectors of TBRF and LB *Borrelia* (PAROLA & RAOULT, 2001). Until 2012, 896 tick species are defined and three families are recognized: two major families of the Argasidae (193 species) and the Ixodidae (702 species) and a third one, the Nuttalliellidae (1 species) (YAKHCHALI et al., 2012). As shown in Figure 3, the Argasidae ticks are characterized with flexible cuticle (soft bodied) while species of the Ixodidae family possess a sclerotized dorsal shield or scutum (hard bodied). Among Argasidae family, the genus *Ornithodoros* comprises the largest number of species (n=112) (GUGLIELMONE et al., 2010). Of them, *O. tholozani* (Figure 3A) serves as the principal vector for *Bp* with a large distribution of regions overlapped by TBRF cases in Middle East, Central Asia, and India and Kashmir. However, the occurrence of *O. tholozani* varies in these areas (ASSOUS & WILAMOWSKI, 2009; MANZANO-ROMÁN et al., 2012). The genus *Ixodes* (243 species) in the Ixodidae family is most vital of vectors that transmit zoonotic pathogens with significant impact on human and veterinary health (GUGLIELMONE et al., 2006; YAKHCHALI et al., 2012). Four predominant species of *Ixodes* ticks (Table 1) have been revealed as competent vectors for LB organisms. In North America, *Bb*ss was transmitted by *I. scapularis* (the deer tick) in the northeastern and upper midwestern USA and in Canada, whereas *I. pacificus* predominately presents in western USA along the Pacific coast. *I. ricinus* (the castor bean tick, Figure 3B) transmits LB agents in Europe and Asia while *I. persulcatus* is endemic in Asia (PIESMAN & GERN, 2004; STANEK et al., 2012).

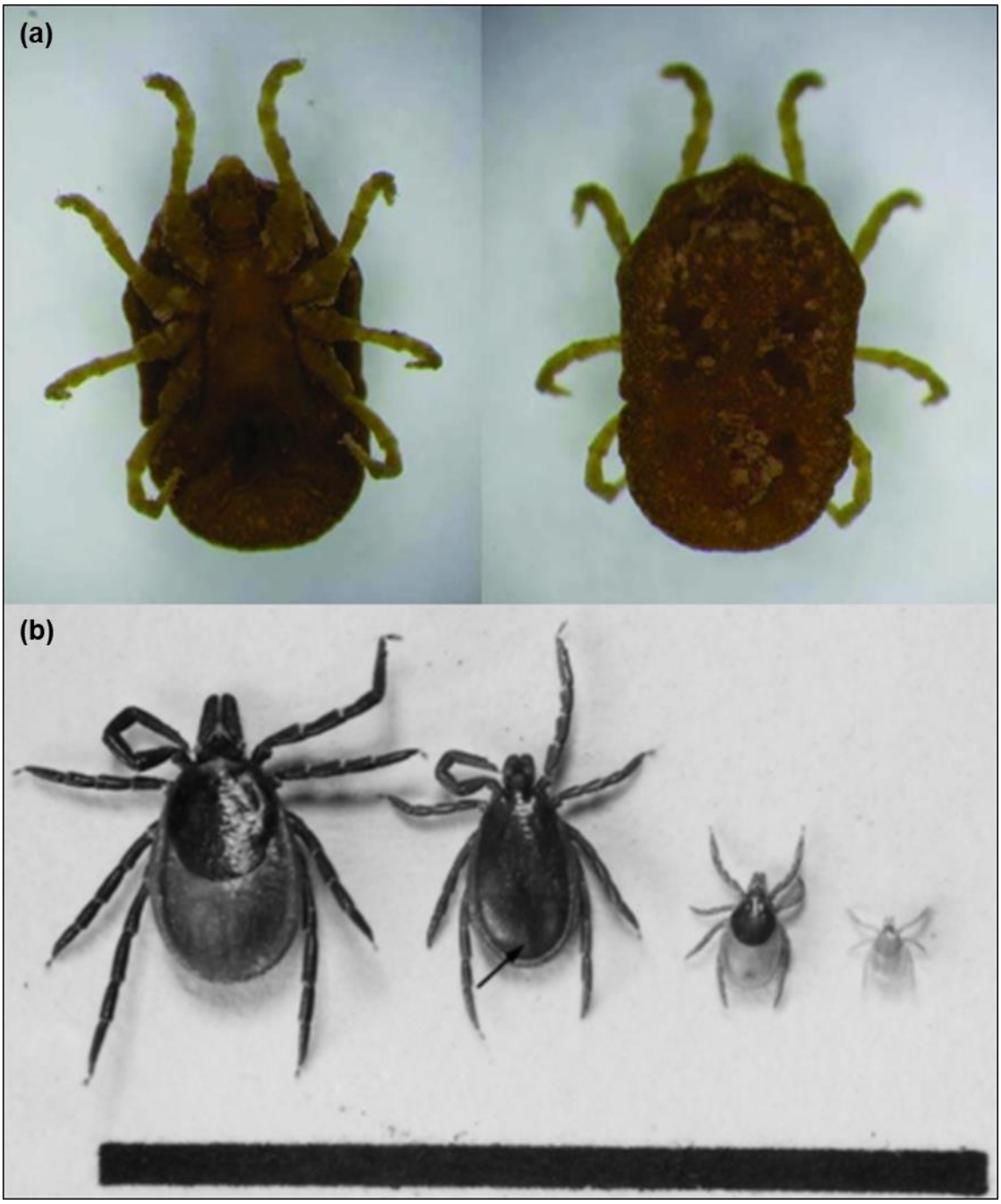

Figure 3: Soft and hard bodied ticks

(a) Soft tick of species *O. tholozani*, left: ventral view; right: dorsal view. **(b)** Three stages of unfed hard tick *I. ricinus*, left to right: adult (female), adult (male), nymph, and larva (bar, 1 cm). Scutum (arrow) covers entire dorsal surface in male, whereas it is confined to anterior part of body in other stages. Note the three pairs of walking legs in larva.
Cited from references (PAROLA & RAOULT, 2001; ASSOUS et al., 2006)

Both soft and hard ticks take blood meals to reproduce, develop, and to complete their life cycle that comprises three stages: larva, nymph, and adult (male and female) (PAROLA & RAOULT, 2001). Before molting to adult, soft ticks may pass through several nymph stages (2 to 5 depending on the tick species; Figure 4). The nymphs feed briefly (minutes to hours) and repeatedly (may up to 6 times) on the same or multiple hosts (BASU & CHARLES, 2017). Differing from soft ticks, the long/slow feeding (2 - 4 days) ixodid ticks have a unique nymphal stage, which means that these vectors feed only once at each stage on various hosts (Figure 5) (RANDOLPH, 1993). After each blood meal, soft ticks drop to molt and hide in their habitats such as the cracks and crevices of the houses, animal burrows, or just below the soil surface. Usually, they emerge at night to feed upon the host while asleep (PAROLA & RAOULT, 2001; BOGITSH et al., 2013). Interestingly, many hard ticks are highly susceptible to drying conditions. Hence, they prefer to locate on or near the soil surface with vegetation with a relative humidity of minimum 80% (RANDOLPH, 1993; CORTINAS et al., 2002; STANEK et al., 2012). Most hard ticks spend more than 95% of their lives on or just below the ground surface digesting the blood meal, molting, in diapause or seeking a host (RANDOLPH, 1993; PIESMAN & GERN, 2004). The life span of hard ticks is generally 2 - 3 years through a life cycle (Figure 5), because adult female ticks feed and lay clutches of eggs only once and die. However, like nymphs, adult females of soft ticks are also capable of taking blood meal for several times. Moreover, soft female ticks lay numerous batches of eggs multiple times during their lifetime (TYSON & J., 2009b). In particular, soft adult ticks can survive 5 - 10 years even with prolonged periods of starvation (DWORKIN et al., 2008; LOPEZ et al., 2016).

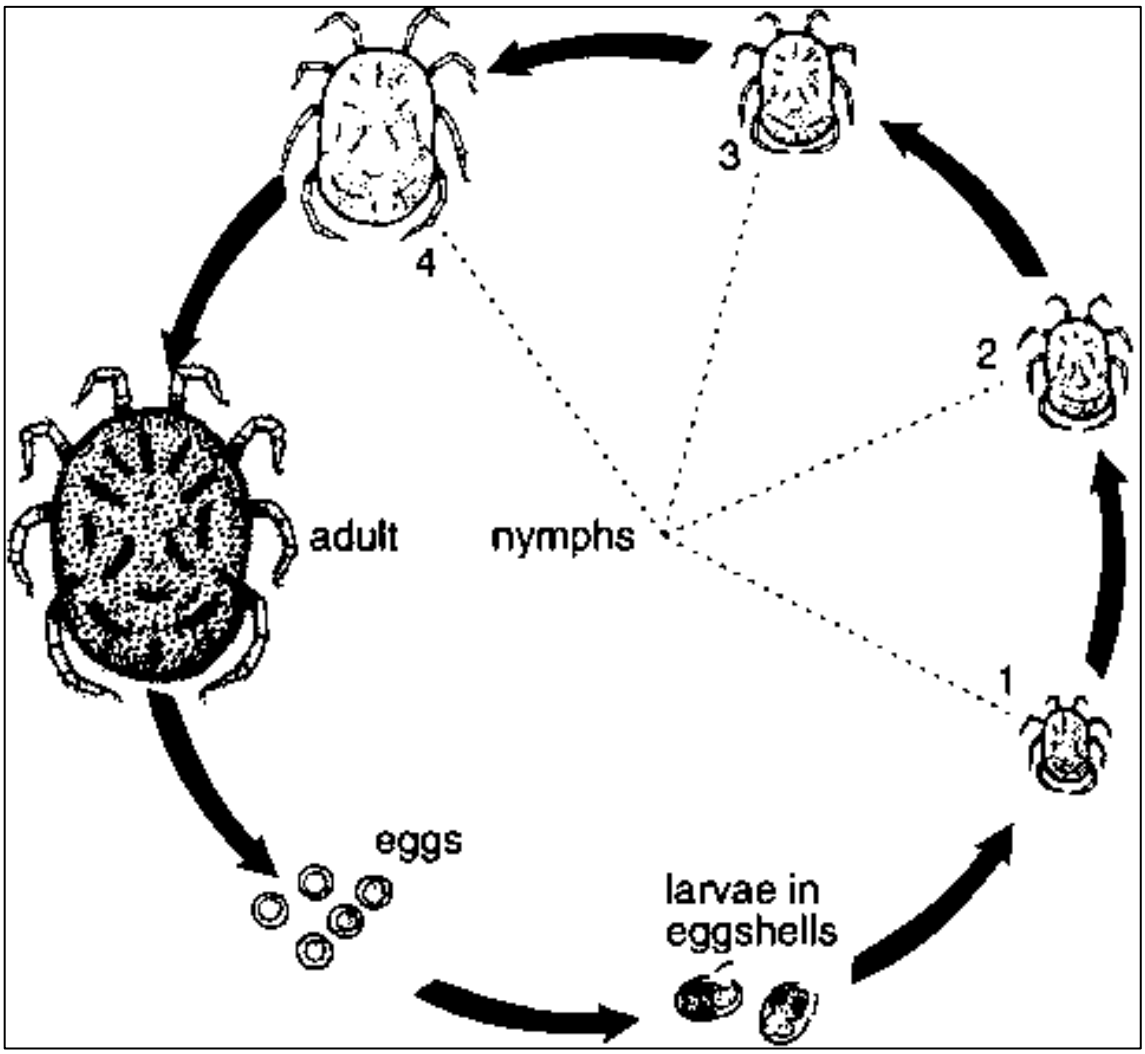

Figure 4: General soft tick life cycle

The example is tick of *O. moubata* species transmitting *B. duttonii* in Africa and Asia. Nymphal instars vary with species.
Cited from WHO (WHO, 1997)

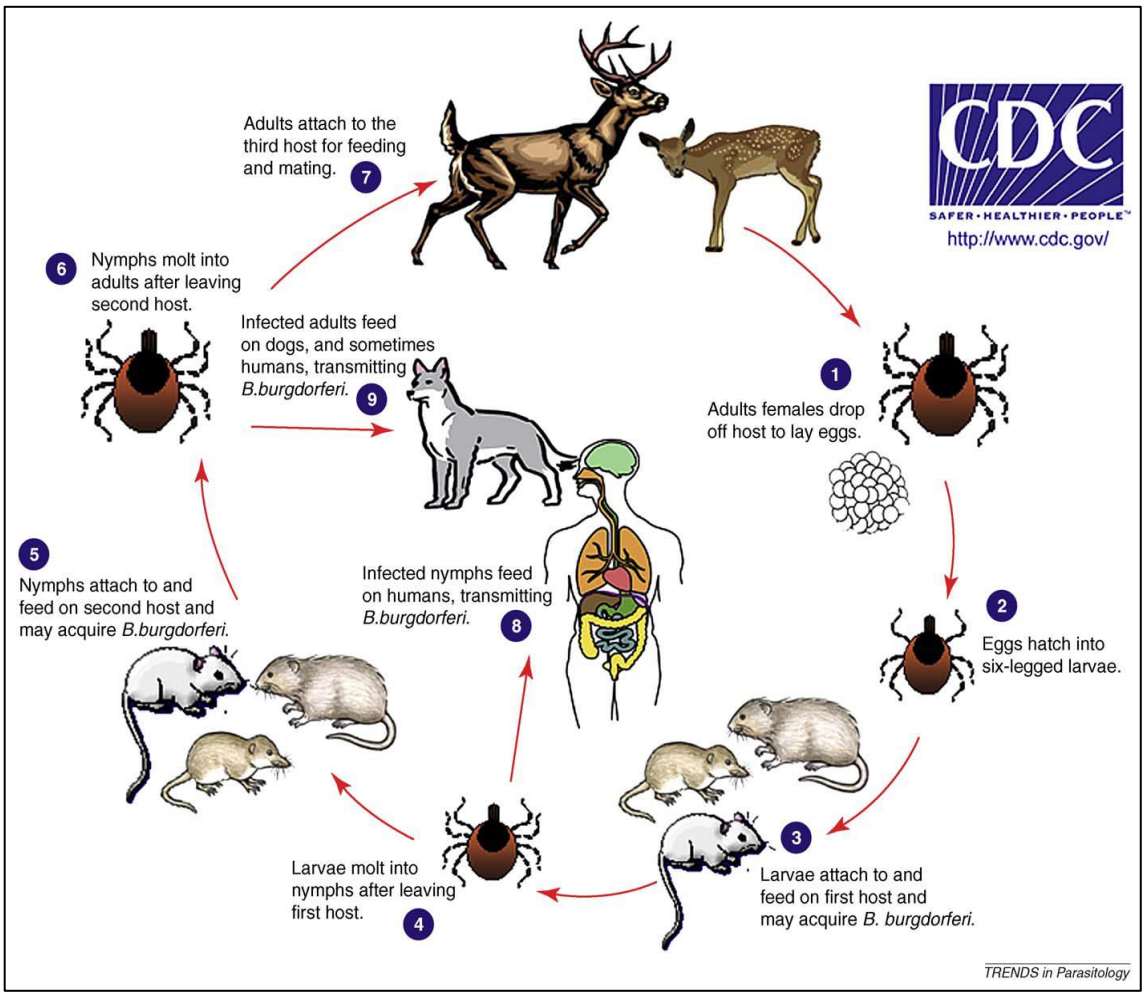

Figure 5: Transmission cycle responsible for maintaining *B. burgdorferi*[2] in tick populations and allowing infection of humans and dogs

Briefly, larvae emerge from eggs laid by female adult in spring, take blood meal upon the first hosts of small vertebrate mammals which may be already infected with borreliae and transfer the bacteria to the fed larvae. Larvae molt in spring of the following year into nymphs that are responsible for spreading the majority of infections via saliva to the second reservoir hosts, humans included. Nymphal ticks are endemic during spring and summer, with a peak activity occurring in late summer. Later, nymphal ticks develop into adults which feed upon the third hosts, including dogs, and reproduce in fall or even in winter.
Cited from Little et al. (LITTLE et al., 2010)

2.3.2 Life cycle of TBRF and LB organisms

TBRF and LB spirochetes cycle through their mammalian reservoirs and tick vectors. The opportunities of transmitting spirochetes vary between soft and hard species because of their contrasting feeding performances (KADA et al., 2017). Characteristically, uninfected larvae acquire borreliae through blood meal from infected rodents or other small mammals. By the

[2] Namely, species of *Bb*ss in North America

time the larvae molt to the nymphs, borreliae are transmitted from infected nymph to uninfected animals during blood meal. Infected animals serve as new reservoirs for continuing the *Borrelia* transmission cycle (Figure 5). After molting to the adults, adult ticks are able to spread borreliae and/or acquire borreliae through blood meal between infected and uninfected animals. Generally, transmission frequency of LB borreliae is influenced by seasonal and environmental conditions (e.g., temperature, relative humidity, and the photoperiod), which can decide the activities of ixodid ticks. The soft ticks, however, have no seasonal preference (PAROLA & RAOULT, 2001). Therefore, the prevalence of TBRF *Borrelia* infection arises more often in summer due to increased human outdoor activities in tick-infested areas (TALAGRAND-REBOUL et al., 2018).

TBRF organisms are mostly horizontally transmitted between *Ornithodoros* ticks and different animals. Also, a transovarial transmission (TOT) of TBRF borreliae exist via the adult's eggs to the offspring. TOT of LB spirochetes, however, is rare or non-existent. Regularly, LB organisms are maintained in the transstadial transmission during molting progress of *Ixodes* spp. ticks: from infected larva to nymph and/or from infected nymph to adult (MAGNARELLI et al., 1987b; RICHTER et al., 2012; ROLLEND et al., 2013). Upon hard tick attachment to susceptible animals, various secretions from the salivary glands facilitate and support the spirochete transmission. There salivary secretions include cement, enzymes, vasodilators, and anti-inflammatory, antihemostatic, and immunosuppressive substances (PAROLA & RAOULT, 2001). Transmission of *Borrelia* spirochetes by soft ticks is faster than hard ticks and mainly through the saliva secretion at nymphal stages or via the coxal fluids from coxal organs of adult ticks throughout the short feeding time (SCHWAN & PIESMAN, 2002).

2.3.3 Reservoirs and hosts

In general, soft ticks are believed to serve as the natural vertebrate reservoirs of TBRF organisms (HOOGSTRAAL, 1985; PAROLA & RAOULT, 2001). *O. tholozani,* is able to transmit *Bp* and have a wide range of mammalian hosts including humans, sheep, goats, camels, cattle, porcupines, hedgehogs, foxes, jackals and rodents (HOOGSTRAAL, 1985). Besides soft ticks, other animals have been also implicated as reservoirs of *Bp*. These animals include bats in Jordan and Central Asia, and rock hyraxes (*Procavia capensis)* in Israel and the West Bank (DE ZULUETA et al., 1971; VASIL'EVA et al., 1990; KLEINERMAN et al., 2018). Recently, reports of natural *Bp* infection in cats and dogs (including a young puppy)

from Israel and Iran suggested that domestic animals are possibly transient reservoir hosts of this bacterium (BANETH et al., 2016; SHIRANI et al., 2016). Soft ticks have no host tropism possibly due to their inhabiting environment and an adaption of natural selection (TALAGRAND-REBOUL et al., 2018). Also, an animal reservoir may not be necessary to complete *Bp* life cycle because of the presence of transovarial transmission (BURGDORFER & VARMA, 1967). Furthermore, an infected *O. tholozani* tick is able to survive impressively long for 10 years or even more without feeding. This long time lasting may have two important roles: as the vector and as the original natural reservoir (GOUBAU, 1984; ASSOUS & WILAMOWSKI, 2009; BANETH et al., 2016). In a result, animals or humans in the life cycle of this tick may only be the source of a blood supply (BANETH et al., 2016).

Ixodid ticks at different life stages may feed on a wide diversity of mammals, birds and reptiles (Table 1) that differ dependening on the geographical distributions. In Europe, *I. ricinus* is the main tick biting various vertebrates with over 300 species. However, only a few of them, such as certain strains of mice, voles, rats and shrews have been detected as reservoirs for *Bb*sl (ANDERSON, 1991; GERN et al., 1998). Garden dormice (*Eliomys quercinus*) in France and edible dormice (*Glis glis*) in Germany were observed parasitized with *Ixodes* spp. ticks and showed their possible roles as borrelial reservoirs (MATUSCHKA et al., 1994; MATUSCHKA et al., 1999). Some rodents like grey squirrels, sheep in the UK and red squirrels in Switzerland are associated with *Bb*sl (CRAINE et al., 1997; OGDEN et al., 1997; HUMAIR & GERN, 1998). In North America, the contribution of the white-footed mouse (*Peromyscus leucopus*) is chiefly substantial as the primary reservoir for *Bb*ss (LANE et al., 1991; ORLOSKI et al., 2000). White-tailed deer, other various species of deer (e.g., elks), and lizards apparently serve as hosts for *I. scapularis* and *I. pacificus* ticks. Particularly, adult *Ixodes* spp. ticks mate on the deer but these animals do not actually act as reservoirs for spirochetal transmission (PIESMAN & GERN, 2004). Some passerine birds (canary finches) may migrate *Ixodes* spp. ticks to new locations and may also act as reservoirs for circulating *B. garinii* and *B. valaisiana* in Europe and *Bb*ss in the USA (OLSEN et al., 1996; PIESMAN & GERN, 2004). In the aspect of veterinaty health, canine infection with *Bb*sl is often associated with infestion of adult ixodid ticks (Figure 5) (KRUPKA & STRAUBINGER, 2010). Humans are considered as accidental hosts for both TBRF and LB organisms (LOPEZ et al., 2016).

3 Pathogenesis of TBRF and LB organisms in mammalian host

3.1 Tick-assisted immune evasion

A successful infection relies on blood meal by infected ticks and a succeptible host. The first step in the infection cascade is the tick crawling to the host skin seeking a safe attachment site (VERHAEGH et al., 2017). Mechanically, the infected vector cuts a hole in host's skin, enabling the rapid (soft tick) or slow (hard tick) delivery of borrelial organisms into the mammal/bird/reptile (BOYLE et al., 2014; STEERE et al., 2016). Biochemically, multifunctional tick saliva or coxal fluids provide adaptive advances in tick feeding and transmission of spirochetes. These vasodilatory molecules secreted by the feeding tick, although most of them are different between soft and hard ticks, can inhibit blood-coagulation and platelet aggregation pathways. These mechnisms allow more blood circulation at the cutaneous bite site and make contribution to the infectivity of the bacteria (KAZIMÍROVÁ & STIBRANIOVA, 2013). Some proteins derived from ixodid tick saliva such as tick salivary protein 20 (Salp20), Salp15, Salp25D are more directed to inactivate the host's innate and adaptive immune pathways including the complement system, $CD4^+$ T cell activation (DAS et al., 2001; ANGUITA et al., 2002; HOURCADE et al., 2016). A large family of *Ixodes scapularis* anticomplement (Isac) proteins and Isac-like family of proteins such as *Ixodes ricinus* anticomplement (Irac) I, Irac II, and IxAC-B1 through 5 have the function to inhibit the host's alternative complement pathway (VALENZUELA et al., 2000; DAIX et al., 2007; COUVREUR et al., 2008). Furthermore, saliva of the hard tick inhibits the Toll-like receptors (TLRs)-induced cytokine responses in keratinocytes as well as on dendritic cells (DCs) (VERHAEGH et al., 2017). Regarding to argasid vectors, however, only *O. moubata* complement inhibitor (OmCI) has been recognized to date. The salivary lipocalins expressed by the tick of *O. moubata* directly bind C5, by which OmCI can inhibite the classical and alternative complement pathways (TYSON & J., 2009a; STONE & BRISSETTE, 2017). Nevertheless, all these mechanisms in turn facilitate the spirochetes to establish the infection in a new host milieu (PAL & FIKRIG, 2003; VERHAEGH et al., 2017).

3.2 Adaptation of borreliae during transmission

Soft ticks of *Ornithodoros* spp. take blood meals as fast as within a few seconds or minutes. For example, ticks of *O. tholozani* and *O. hermsii* attach 10 - 20 min and 15 - 90 min, respectively, for repletion after encountering their hosts. During the short feeding time,

pathogenic TBRF spirochetes can be efficiently transmitted from a tick vector to the new host (SCHWAN & PIESMAN, 2002; ASSOUS & WILAMOWSKI, 2009). Recent studies have described that deposition of *B. turicatae* into the bite site is a rapid event within 15 s of tick attachment during the blood meal. Particularly, their findings suggested that spirochetes disseminate into the host blood during the short time required for tick engorgement (BOYLE et al., 2014). Thus, it is proposed that a preadaptation of TBRF borreliae is likely established in the tick salivary glands. During the preadaptation period, pathogens can express some proteins against innate immunity in order to enter the vertebrate host by a rapid transmission, e.g., variable tick protein (Vtp) synthesized by *B. hermsii* (SCHWAN & PIESMAN, 2002; RAFFEL et al., 2014; LOPEZ et al., 2016).

Lyme spirochetes are well-identified pathogens that alters the expression of Osps (Figure 6) over the course of its life cycle between the tick vector and mammalian host. Various Osps are crucial antigens in cellular physiology and act directly in pathogenesis in mammals (FRASER et al., 1997; SINGH & GIRSCHICK, 2004). OspA is expressed as LB spirochetes enter the tick vector during the uptake of an infected blood meal. This protein remains a key surface antigen during bacterial colonization in the midgut of unfed ticks (BATTISTI et al., 2008). During the slow engorgement process of infectious tick feeding (2 - 4 days of the complete 5- to 8-day attachment period), spirochetes that undergo ambient temperature and pH changes (from 23 °C, high pH to 37 °C, low pH) migrate from the midgut to the salivary grands (TEMPLETON, 2004). Concurrently, these bacteria multiply to increase their quantity and downregulate the expression of OspA (particularly) and OspB (DE SILVA & FIKRIG, 1995; ANGUITA et al., 2003). Another surface protein, OspC, is highly upregulated and plays a pivotal part in spirochetal dissemination from tick midgut to the host dermis (GILMORE & PIESMAN, 2000; TILLY et al., 2008). Since spirochetes with OspC are able to invade the tick's salivary glands and bind Salp15, prevention of complement- or phagocytosis-mediated killing and further recognition by antibodies (especially immunoglobulins M, IgM) is beneficial for LB organisms to survive at early stage of mammalian infection (SCHUIJT et al., 2008; CARRASCO et al., 2015). Studies reviewed by Verhaegh et al. show that deleting or overexpressing OspC results in quick clearance of borrelial organisms from the host (VERHAEGH et al., 2017). However, OspC production is not required for spirochete persistence in the host once the infection is established (TILLY et al., 2006). Nevertheless, these Osps, especially OspA and OspC as identified, are able to bind vector or host molecules. This mechanism protects the LB organisms against the innate

immune response during the initial stage of host colonization/invasion (RUDENKO et al., 2016).

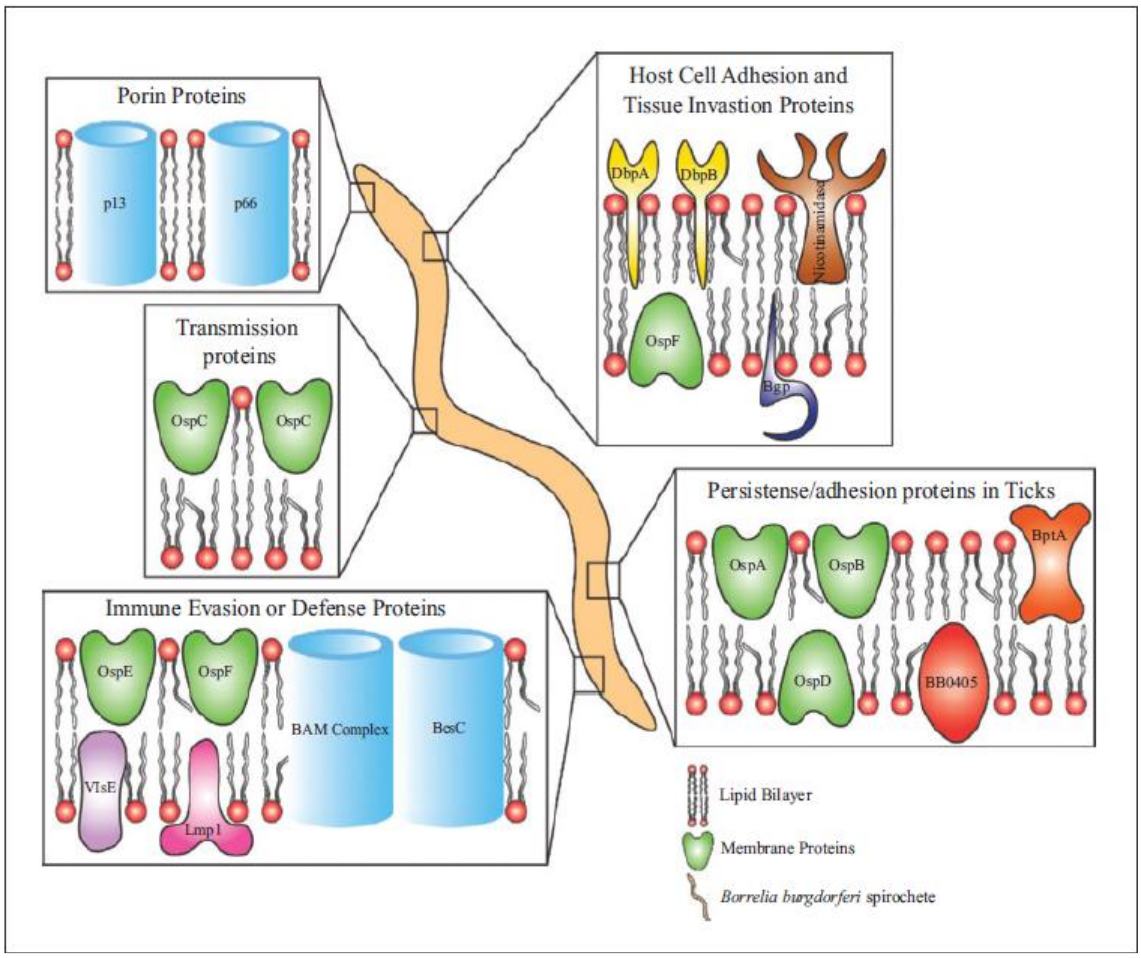

Figure 6: Overview of the LB spirochete and its Osps ordered according to their (predicted) function

Cited from Verhaegh et al. (VERHAEGH et al., 2017)

3.3 Establishment of early infection

Although the precise number of delivered TBRF spirochetes through the tick saliva remains unknown, one can speculate that it is low due to the very short (seconds to minutes) feeding course of *Ornithodoros* spp. ticks. Successful infections have been established in laboratory animals (ticks included) under different settings with variable infection doses and inoculation types. A single spirochete of *B. recurrentis* var. *turicatae*, *B. duttoni*, and *B. hermsii* is sufficient to produce an effective infection in rats (IP inoculation), soft ticks of *O. moubata* (capillary tube), and mice (IP inoculation), respectively (SCHUHARDT & WILKERSON, 1951; GEIGY & SARASIN, 1958; STOENNER et al., 1982). Motile

spirochetes appear in the blood of white mouse as soon as 1 h after IP injection of "a large inoculum" of a RF species "*Borrelia erratici*" (EIDMANN et al., 1959; BARBOUR & HAYES, 1986). Kinetic studies on the spirochete populations have confirmed mild to high-level bacteremia in the blood of immunocompetent animals one day after tick attachment, ID, and IP inoculation of *B. turicatae*, *B. persica*, and *B. hermsii*, respectively (BOLZ et al., 2006; LOPEZ et al., 2014; SCHWARZER et al., 2016). Therefore, TBRF spirochetes occupy a feature that they can pass rapidly from the tick bite site to the blood of the vertebrate host. Consequently, as early as the first 3 to 5 days after inoculation, TBRF pathogens that have survived in and adapted to the bloodstream can multiply and produce severe spirochetemia at a density of approx. 10^6 - 10^8 organisms per ml blood (SOUTHERN & SANFORD, 1969; BARBOUR & GUO, 2010; LOPEZ et al., 2016).

In contrast to TBRF, even low-level spirochetemia is rarely seen in humans or animals with LB borreliae infections (BARBOUR & HAYES, 1986). In fact, the LB spirochetes remain locally in the mammal's dermis for few days before their colonization in different tissues, including the distant skin sites or such organs as joint and heart where they can induce inflammatory responses (SHIH et al., 1992; ANGUITA et al., 2003; GRILLON et al., 2017). In the natural reservoir hosts, infections with LB organisms generally do not induce obvious disease manifestations, because spirochetes have co-evoluted immune evasion strategies with the mammals (TRACY & BAUMGARTH, 2017). The majority (95%) of the exposed dogs in the USA develop no clinical signs and if they become ill, arthritis, fever, anorexia, and lymphadenopathy are most common symptoms (LITTLE et al., 2010). Although humans are believed as occasional hosts and do not contribute to spreading pathogens to another host, they are susceptible to *Bb*sl infection and would experience severe disease complications in some cases (RADOLF et al., 2012). The earliest and most common clinical manifestation of LB in humans is characterized with EM, a red skin rash at the tick bite site (Figure 7), occurring in approx. 80% and 90% of patients in the USA and Central Europe, respectively (STEERE & SIKAND, 2003; STANEK & STRLE, 2018). If left untreated, EM may persist and expand over weeks to months, their diameter ranging from a few centimeters (≥ 5 cm) to more than a meter (Figure 7) (STANEK & STRLE, 2018). Multiple EM are described with the presence of a secondary or more lesions similar in morphology to the initial solitary lesion but smaller in the size criteria (STANEK & STRLE, 2018). As mentioned in an investigation, the expanding EM seems to represent the advancing front of a wave of the spirochetal organisms "dermatogenously" migrating outward from the deposition site in the

skin of the human host (SHIH et al., 1992). Pathogenically, the inoculated spirochetes initially sense the pattern recognition receptors (PRRs) such as TLRs and NOD[3]-like receptors (NLRs) on DCs and sentinel macrophages in the cutaneous bite site of the vertebrate host. Accordingly, certain pro-inflammatory molecules including type 1 interferons (IFNs) and inflammatory chemokines and cytokines are released by the host immune cells with the engagement of TLRs. Also, infiltration of immune cells such as T cells ($CD8^+$ and $CD4^+$ cells), macrophages, plasmacytoid, monocytoid DCs, and neutrophils have been identified in biopsies of human EM skin lesions (RADOLF et al., 2012). Therefore, EM by LB patients is thought to be induced by both innate and adaptive immune response to the live and spreading spirochetes (BORCHERS et al., 2015).

[3] NOD: nucleotide-binding oligomerization domain

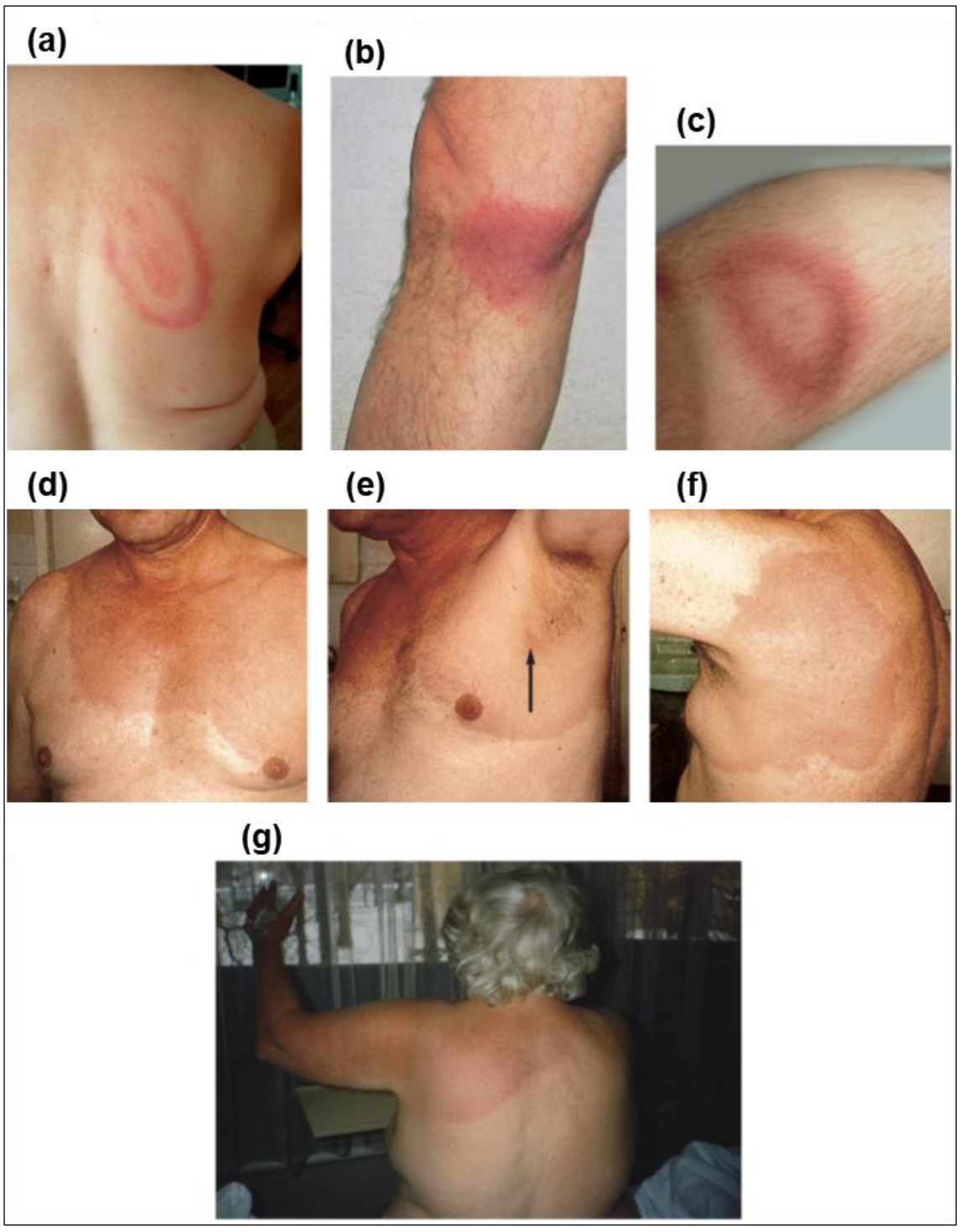

Figure 7: Erythema migrans (EM) skin lesions

(a) EM shoulder, (**b)** EM knee pit, (**c)** EM lower leg, a day after onset of treatment, (**d-f)** EM~9 weeks after onset; tick bite in the axilla, (**g**) EM started at the breast 6 months before
Cited from Stanek and Strle (STANEK & STRLE, 2018)

3.4 Susceptibility to host complement system

Complement represents a central component of innate immunity. It functions as a well-organized network comprising the classical, alternative, and the lectin pathway (STONE & BRISSETTE, 2017). The alternative pathway is triggered independently of antibodies and therefore serves as a primary host defense against borrelia infection before any involvement of humoral immune response. Particularly, a key regulatory factor H (FH; 150-kDa protein) is involved in the alternative pathway of complement activation (PAL & FIKRIG, 2003; WOODMAN et al., 2009). However, various native proteins expressed by *Borrelia* spp. organisms are able to inhibit/regulate complement activation. According to their mechanisms, these native proteins are collectively termed as factor H binding proteins (FHBPs) or complement regulator-acquiring surface proteins (CRASPs) (KRAICZY & STEVENSON, 2013). TBRF borreliae have successfully established various sophisticated mechanisms to overcome innate immunity, mostly complement system (RÖTTGERDING et al., 2017). At least for *B. hermsii* resistance to complement mediated killing activity has been demonstrated (BHIDE et al., 2009). *B. miyamotoi* was described with persistence in the human serum, indicating that this bacterium grasps determinants to evade killing by complement (TEEGLER et al., 2014; WAGEMAKERS et al., 2014). More recently, complement binding and inhibitory protein A (CbiA) has been identified and found to directly inhibit both the initiation of classical pathway of complement and the assembly of the terminal complement complex (RÖTTGERDING et al., 2017). Taken together, TBRF spirochetes probably have evolved strategies to resist (successfully) the complement activation for their survival in the host (MERI et al., 2006; ROSSMANN et al., 2007). In addition, resistance to complement strengthens the transmission, survival, and dissemination of the *Borrelia* spp. in mammalian and rodent reservoir hosts (KURTENBACH et al., 2002).

Regarding to LB organisms different OspE/F-related proteins (Erp) (Figure 6), which are known collectively as CRASPs, are synthesized to inhibit host complement during early infection phase (PAL & FIKRIG, 2003). Especially the OspE protein has been demonstrated to bind the host FH protein (HELLWAGE et al., 2001). However, different isolates and genospecies of LB vary in their susceptibility to normal human serum and are therefore classified as complement-resistant (most isolates of *B. afzelii*), complement-sensitive (most *B. garinii* isolates), and intermediate complement-sensitive (*Bb*ss isolates) (KRAICZY et al., 2001; DE TAEYE et al., 2013). There is evidence that these three pathogenic genospecies are related to diverse clinical manifestations in humans: LA with *Bb*ss, LNB with *B. garinii*,

and ACA with *B. afzelii* (COIPAN et al., 2016). Differences in the complement susceptibility may play a part in the pathogenesis of the disease; whether or not complement resistance is a virulence factor of LB spirochetes needs to be confirmed. Nonetheless, one must consider that *B. afzelii* has the capacity of persistence in LB patients with ACA over the years. This may suggest a higher pathogenicity of complement-resistant *B. afzelii* isolates to the skin (KRAICZY et al., 2001).

3.5 Immune evasion by borrelial antigenic variations

Borrelial spirochetes experience an important change in environment when they are transmitted by a tick vector, which has no antibody-based immune system, to the vertebrate hosts, of which many are warm-blooded and have the capacity of responding uniquely to many different types of foreign substances and cells (BARBOUR, 1990). To face the vertebrate's adaptive immune responses, *Borrelia* spirochetes achieve persistence through multiphasic antigenic variation (BARBOUR, 1990).

3.5.1 Variable major proteins of TBRF *Borrelia*

Human infection by TBRF *Borrelia* is characterized with recurring episodes of fever, which is consistent with the high-level spirochetemia in the blood (Figure 8) (DWORKIN et al., 2008). Immunity of the infected mammals is therefore stimulated to generate borreliae-specific antibodies of IgM. Once the density of antibodies is high enough, the initial spirochetal wave is rapidly eliminated from the bloodstream (Figure 8). Borrelial cells are not detectable in the blood resulting in afebrile period (BARBOUR, 1990; STONE & BRISSETTE, 2017). However, the lower number of spirochetes (10 to 1,000 cells/ml) in the blood switches to produce another antigenic serotype that is not recognized by the host immune response. As a result, a new population of bacteria emerges in the blood (MEIER et al., 1985; BARBOUR & HAYES, 1986; DAI et al., 2006). Accordingly, old populations are being replaced by new serotypes that have been formed in the meantime (Figure 8). The serotype-specific antigens are surface lipoproteins of the spirochete outer membrane and have been referred to as variable-major-proteins (Vmps). The existing two different classes of Vmps are the variable large proteins (Vlps) and the variable small proteins (Vsps) of about 38 kDa and 23 kDa, respectively (BARBOUR, 1990; CADAVID et al., 1997; CADAVID et al., 2001). Up to now, the mechanism of antigenic variation has been described in the following species: *B. hermsii* (PLASTERK et al., 1985), *B. turicatae* (RAS et al., 2000), and more recently in *B. miyamotoi* (WAGEMAKERS et al., 2016). Regarding to *Bp*,

knowledge of its genetic mechanisms at the level of Vmps is still limited, although Vmp sequences have been identified (e.g., Vlp18 under NCBI accession number: WP_024653159) (SCHWARZER et al., 2016). Nevertheless, immune evasion of TBRF spirochetes due to the multiphasic antigenic variance allows a longer lasting and repeated spirochetemia (Figure 8). This strategy gives the chance of increasing the horizontal transmission of TBRF borreliae in nature, because recurrent presence of these organisms in the blood increases the likelihood of an infection of ticks that suck blood in short time (minutes) (PLASTERK et al., 1985; SCHWAN & PIESMAN, 2002).

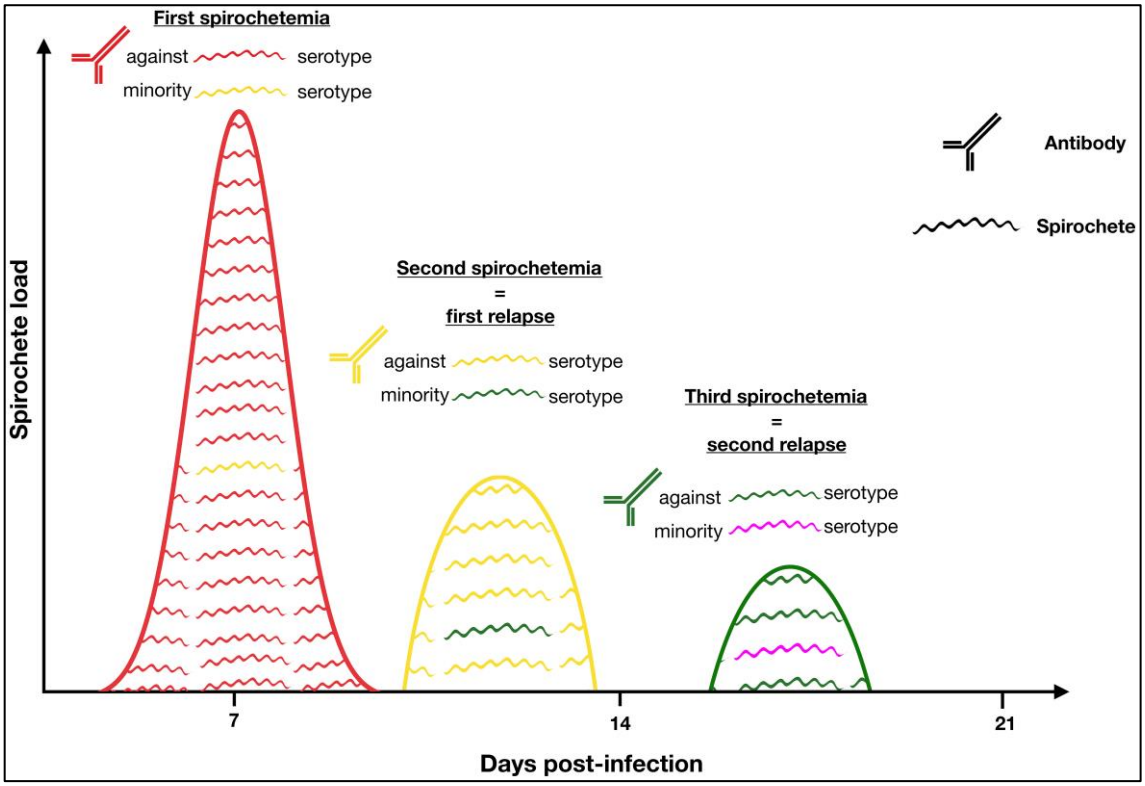

Figure 8: TBRF increases its persistence in blood by shifting the surface protein Vmp

When antibodies are induced against the first serotype (red), all borreliae expressing this serotype are killed by Vmp-specific antibodies. Only those that have shifted to a second serotype (yellow) survive and proliferate to cause the first relapse. This combat continues until the host dies or the borrelial organisms are eradicated from the blood. Antigenic variation is the mechanism initiating the recurring fever, which gave the disease its name. Relapses rarely involve one, single serotype. Cited from Talagrand-Reboul et al. (TALAGRAND-REBOUL et al., 2018)

3.5.2 Variable major protein-like sequence of LB *Borrelia*

After the establishment of *Bb*sl infection, the evasion of bactericidal antibodies becomes crucial for borrelial survival. For this purpose, the spirochetes again alter rapidly and continually the surface antigen epitopes by downregulation of OspC and simultaneous upregulation of VlsE protein, i.e., Vmp-like sequences (Vls) system (NORRIS, 2006; TILLY et al., 2008; STEERE et al., 2016). As each Osp is present as a single-copy locus, genetic variation is seen at the population level. Therefore, a single spirochete cannot produce various OspC types *in situ* (STONE & BRISSETTE, 2017). In contrast, VlsE encoded by *vls* gene can undergo extensive antigenic recombination. This is a significant variation system to help LB organisms evade the killing by host antibodies and maintain persistent infection in mammals (ZHANG et al., 1997). The Vls system (Figure A1) comprises around 16 *vls* cassettes (the precise number differs by strain) and one expression locus, *vlsE*. All known *vls* cassettes are sited on the same linear plasmid (lp28-1) in close proximity to but in the opposite orientation of *vlsE* (ZHANG et al., 1997). Transcription of *vlsE* occurs through the random recombination of different segments of *vls* cassettes rather than recombination of an entire, single *vls* cassette. Thus, in contrast to TBRF borreliae that harbor a predominant serotype, recombination steps occurring to LB spirochete may generate thousands of sole VlsE variants but with a similar protein size (~ 36 kDa) after translation (STONE & BRISSETTE, 2017). Accordingly, shifted epitopes and confounding efforts by humoral immune response (antibodies IgG) to keep up with the sequence variation can be harbored in the infected mammals (MCDOWELL et al., 2002; NORRIS, 2006). As early as 4 days after *Bb*sl infection in mice, the recombination events have been identified and seem to occur continuously during infection (MCDOWELL et al., 2002). Interestingly, mutants that express non-variable VlsE are unable to re-infect animals that have been previously infected with *Bb*sl, whereas spirochetes that express variable VlsE can (ROGOVSKYY & BANKHEAD, 2013). However, unlike TBRF, recurring spirochetemia corresponding to recurrent episodes of high fever are not seen during LB organism infections.

3.6 Persistence of *Borrelia* infection in mammalian host tissues

3.6.1 Residual brain infection of TBRF spirochetes

Antigenic variation facilitates the *Borrelia* infection in the mammals. When TBRF spirochetes are no longer measurable in the blood circulation, they may still be found in distant organs such as spleen, liver, kidneys, bone marrow, eyes, and the central nervous

system (CNS) of infected mammals. In particular, brain tissue harbors consistently persistent infections (BARBOUR & HAYES, 1986; CADAVID & BARBOUR, 1998; DWORKIN et al., 2008). Borreliae have also been recovered from the cerebrospinal fluid (CSF) of humans with RF (CHUNG, 1938; ANDERSON & ZIMMERMAN, 1955). Recently, *Bp* was found to be persistent in the brain tissues of infected immunocompetent mice while at the same time the spirochetes were not detectable in blood samples from the same animals (SCHWARZER et al., 2016). Residual brain infection has been found in different experimental animals or animals in nature (DIATTA et al., 1994; CADAVID & BARBOUR, 1998). In some cases, brain infection has been found to remain for up to 3 years after spirochete inoculation. This indicates that these borreliae maintain a population large enough to perpetuate the infection in the brain, yet small enough to avoid host immune responses and noticeable tissue damage. Additionally, borreliae recovered from residual brain infection were susceptible to the serum of the animal and could not reenter the blood of that animal. These phenomena also proposed that the immune response was not effectively reaching the organisms in the brain (CADAVID & BARBOUR, 1998). However, immunosuppression could reactivate the infection, inducing bacteremia comparable to that of initial densities in the blood (LARSSON et al., 2006).

3.6.2 Invasion of LB spirochetes in host tissues

Clinically, spirochetemia at the early stage of LB organism infection was often silent in both American and European patients with erythema migrans. Blood culturing for *Bb*sl spirochetes have been conducted with great efforts but the yield was low in early or occasionally later course during disease progression (WORMSER et al., 1998; MARASPIN et al., 2001; LIVERIS et al., 2011; MARASPIN et al., 2011). Instead, following successful host colonization, LB spirochetes have a tendency to reside in host tissues. There the borrelial organisms evade the developing adaptive immunity of the host. Typically, skin and joint, where the host extracellular matrix protein decorin is found, are the immune-privileged sites for LB bacteria (TILLY et al., 2008; BORCHERS et al., 2015). Also, the skin but not the blood of the vertebrate reservoir host is indicated to play a critical part in receiving, hosting, and transmitting the LB pathogens (GRILLON et al., 2017). A large number of genes are selectively expressed on outer membranes of *Bb*sl, which likely contribute to spirochete migration and colonization in target tissues (PAL & FIKRIG, 2003). Among them, decorin-binding proteins (Dbps) such as DbpA and DbpB and fibronectin binding protein BBK32 have been identified with critical roles in LB organism infection within the

mammalian environment (Figure 6) (GUO et al., 1995; GUO et al., 1998; PROBERT & JOHNSON, 1998). The pore protein P66, another *Bb*sl surface antigen, has been reported to have adhesive properties and bind human ß3-chain integrins that are synthesized in a wide range of host location (COBURN et al., 1999). Therefore, multiple adhesin mechanisms through widespread host receptors such as decorin, fibronectin or integrins contribute to the virulence of the LB spirochete and aid the pathogen to establish chronic infection in multiple tissues (PAL & FIKRIG, 2003).

The most significant clinical sign of LB is acute or chronic arthritis (LA) occurring to ~ 10% of all LB patients in the United States. However, the isolation rate of borrelial spirochetes from joint fluid and synovial is very low (STANEK & STRLE, 2018). In Europe, acrodermatitis chronica atrophicans (ACA) remains a slowly progressive lesion that is located primarily on the extensor (acral) surfaces of the extremities. Approximately 20% of LB patients with ACA have a history that is consistent with a preceding spontaneously healed EM lesion. Usually, ACA lesion develops 6 months to 8 years later on this healed EM extremity. Unfortunately, ACA does not resolve spontaneously. *Bb*sl spirochetes have been isolated more than 10 years post onset of this skin lesion from untreated patients with early disease manifestations (EM or early LNB) (ASBRINK & HOVMARK, 1985; STEERE et al., 2016).

3.7 Patterns of *Borrelia* dissemination in mammalian host

3.7.1 Hematogenous dissemination of TBRF borrelia

TBRF spirochetes translocate rapidly into the bloodstream from the tick bite site, multiply and produce cyclic high-level spirochetemia (BOYLE et al., 2014; LOPEZ et al., 2016). In the absence of antibiotic treatment, the cyclic nature of bacteremia in the blood and its correlated clinical sign, recurring fever, can continue even for months (SOUTHERN & SANFORD, 1969). *B. duttonii*, *B. crocidurae*, *B. hispanica* and *Bp* frequently bind or rosette blood erythrocytes, and thus cause erythrocytes to aggregate. This phenomenon can prevent the interaction of spirochetes with host immune cells and thus may enhance persistence of these organisms within the bloodstream (BURMAN et al., 1998; SHAMAEI-TOUSI et al., 1999; TALAGRAND-REBOUL et al., 2018). Along the infection time, motile organisms in terms of *B. hermsii*, *B. turicatae*, *B. crocidurae* and *Bp* spp. have been isolated from the animal brain tissue, although spirochetes are not detectable in the bloodstream (CADAVID et al., 2001; NORDSTRAND et al., 2001; MEHRA et al., 2009; SCHWARZER et al., 2016).

Undoubtedly, these TBRF borrelia, as well as the LBRF species *B. recurrentis*, have an excellent ability to cross the microvessels and tight barriers, such as blood-brain-barrier (BBB) and the dura mater-arachnoid barrier (CADAVID et al., 2001). Moreover, blood transfusion-transmitted infections (TTI) of *B. recurrentis* and *B. duttonii* in humans have been reported (WANG & LEE, 1936; HIRA & HUSEIN, 1979; NADELMAN et al., 1990), indicating that these accidentally injected borrelial organisms are capable of surviving in the bloodstream of the adopter. After IV inoculation in experimental immunocompetent animals, *B. hermsii* and *B. miyamotoi* induced a mild to high-level spirochetemia (BENOIT et al., 2010; POLICASTRO et al., 2013; DICKINSON et al., 2014; KRAUSE et al., 2015). Therefore, these TBRF spirochetes are considered to have predominantly a hematogenous/blood-borne dissemination route. In this way, borreliae enter and leave the bloodstream by invading through the endothelium, followed by a massive spirochetemia and further infection in distant tissues (e.g., brain) (MERI et al., 2006).

3.7.2 Controversial dissemination pathways of LB borrelia

Once transmitted into the dermis of the mammalian host, *Bb*sl spirochetes were shown to disseminate to a variety of remote sites. The pathogens have been detected in skin, blood, CSF, and synovial fluids in humans (BENACH et al., 1983; STEERE et al., 1983; JOHNSTON et al., 1985) as well as in skin, blood, and urinary bladder in rabbits and natural reservoir hosts (*P. leucopus*) (KORNBLATT et al., 1984; SCHWAN et al., 1988). According to some experimental studies on LB spirochete infection, some systematic manifestations especially in skin tissue, joints, nervous system, or heart were induced (STEERE et al., 2016). However, the relationship between the dissemination of spirochetes and the subsequent severity of disease is a complex question. Generally, dissemination through the bloodstream has long been hypothesized as the main route of spreading the borrelia organisms (GOODMAN et al., 1995; WORMSER et al., 1999; OJAIMI et al., 2005). Reports of *B. burgdorferi*-positive blood cultures from LB patients or of blood samples tested positive by polymerase chain reaction (PCR) in early stages of the illness seem to support the view of a blood-borne bacterial spread (BENACH et al., 1983; GOODMAN et al., 1995). However, these reports, which were based on large sample volume of 9 - 18 ml plasma or blood, yielded a very low rate of spirochete-positive culture (≤ 5%) (WORMSER et al., 1998; WORMSER, 2006). Results of such culturing were consistent with an estimate of 0.1 cultivable spirochete per ml blood (WORMSER, 2006). Therefore, the bacterial burden in blood is actually extremely low. Additionally, an in vitro investigation on *B. recurrentis*,

B. duttonii, and *Bb*ss provided evidence that LB pathogens are less immune serum-resistant than the RF spirochetes (MERI et al., 2006), indicating that LB spirochetes are less able to survive in the blood than the RF species.

On the other hand, some authors hold the view that LB borreliae disseminate from the tick bite site through perhaps blood or tissue planes to other distant locations (STANEK et al., 2012). Other authors also stated a "dermatogenous" migration of spirochetes from the deposition site outward to further tissues causing EM skin lesions of the human host (SHIH et al., 1992). Experimental data on the development of persistent LB in dogs clarified that live spirochetes are found more frequently in tissues of the somatic quadrant closest to the tick bite than in tissues further from the site of infection (STRAUBINGER et al., 1997). If the LB borreliae had spread to the joint space through the bloodstream, a random distribution of infection of different joints would have been anticipated. Therefore, this interesting study suggested that the progress of chronic LB is not due to proliferation of borrelia in the bloodstream, but especially due to the migration of spirochetes through various tissues (STRAUBINGER et al., 1997). Additionally, in a separate investigation of humans with LNB, the presence of skin lesion EM on an extremity was also topographically related with motor paresis of that specific extremity (HANSEN & LEBECH, 1992). This finding has also suggested that the spirochetes disseminate through soft tissues. Notably, up to now there is no reported cases of transfusion-transmitted infections by *Bb*sl via donated blood from LB patients, although other transfusion-associated bacteria such as *Babesia microti* and *Anaplasma phagocytophilum*, which are transmitted by the same *Ixodes* spp. ticks, have been documented (LEIBY, 2004; BIHL et al., 2007).

The differences of TBRF and LB species (Table 2) still remain to be further determined, but probably affect the pathogenetic properties of the organisms (BUNIKIS et al., 2004). Mechanisms of dissemination and invasion are important not only for the pathogenesis but also for the development of spirochete persistence in hosts.

Table 2: Comparison of TBRF, LBRF and LB species associated with their pathogenicity

characteristic	TBRF	LBRF	LB
agent	several species	*B. recurrentis*	*Bb*sl[a]
vector	*Ornithodoros* spp. (soft ticks)	*P. humanus* (human body louse)	*Ixodes* spp. (hard ticks)
fever (temperature of ≥ 39 °C)	common	common	rare[b]
fever relapses	multiple	few	no
local skin rash	no	no	common (EM[c])
arthritis	no	no	common[d]
spirochetes in blood smear	yes	yes	no
spirochetemia (cells/ml blood)	from 10^5 to $>10^6$	from 10^5 to $>10^6$	occasionally but low level
neurological involvement	common[e]	rare	common[f]
tissue tropism	brain	brain	skin, joint
serological assay specificity	fair to poor[g]	fair to poor[g]	good to excellent
dissemination pathway	hematogenous[h]	hematogenous[h]	hematogenous or tissue migration[i]
blood transfusion-mediated infection	human and mouse model cases[j]	humans cases[k]	no

[a] typically: *Bb*ss, *B. afzelii*, *B. garinii*
[b] Fever is rarely one of the constitutional symptoms accompanied with the development of erythema migrans at early stage of LB (BORCHERS et al., 2015).
[c] erythema migrans
[d] Arthritis is the most common manifestation of late LB, occurring weeks to months after the initial infection (MURRAY & SHAPIRO, 2010).
[e] common in ≥ 10% of patients with disseminated disease
[f] Lyme neuroborreliosis (LNB) may be associated with early disseminated stage of LB spirochete infection (BORCHERS et al., 2015).
[g] Variable major proteins (Vmps) of RF borreliae during infection may not be produced by the in vitro cultured spirochete that is utilized as a whole-cell lysate antigen in the serologic testing; this may reduce the reactivity of a sample in the IFA and ELISA falsely.

[h] supported by references summarized in chapter **3.7.1**
[i] supported by references summarized in chapter **3.7.2**
[j] supported by references (HIRA & HUSEIN, 1979; NADELMAN et al., 1990; BENOIT et al., 2010; POLICASTRO et al., 2013; DICKINSON et al., 2014)
[k] supported by references (WANG & LEE, 1936; NADELMAN et al., 1990)
Data summarized and modified from references (BARBOUR & HAYES, 1986; CADAVID & BARBOUR, 1998; DWORKIN et al., 2008)

III STUDY HYPOTHESIS AND OBJECTIVE

Prior studies have reported rapid and high-level spirochetemia of *Bp* in the bloodstream of laboratory mice (SCHWARZER et al., 2016). After deposition by ixodid ticks, LB spirochetes multipy locally in the skin during the first few days of infection and subsequently disseminate to remote sites, thus causing clinical manifestations (GRILLON et al., 2017). To the best of our knowledge, we hypothesize that 1) *Bp* spirochetes probably rely on a hematogenous dissemination, like the other well-studied RF species (e.g., *B. recurrentis*, *B. hermsii*, *B. duttonii*, and *B. turicatae*) (MERI et al., 2006; BOYLE et al., 2014; LOPEZ et al., 2016); 2) (most) motile LB spirochetes at the deposition site migrate actively through tissues to further distant tissues and accidentally (only some few of the deposited borreliae) may penetrate the blood vessel into bloodstream. To test these hypotheses, we established a murine anmial model. In this study model, host-adapted *Bp* and *Bb*ss organisms were intradermally (ID) or strictly intravenously (IV) inoculated into immunocompetent animals. If the IV injected spirochetes are capable of multiplying in the bloodstream and transmigrating through the blood vessels, further infection should be established in distant tissues of the immunocompetent animal. Moreover, the immune system should recognize and respond to the multiplying bacteria. Only based on positive results from IV inoculated animals can it be undoubtedly believed that *Bp* and *Bb*ss borreliae disseminate hematogenously.

The objectives of our murine infection model were to:

i) establish an animal model with a specific infection route;
ii) investigate the population kinetics of borrelia organisms in the bloodstream of the immunodeficient and immunocompetent mice;
iii) study whether host-adapted, ID or IV inoculated *Bp* and *Bb*ss disseminate into tissues of immunocompetent mice;
iv) compare the tissue tropism of *Bp* and *Bb*ss in immunodeficient and immunocompetent mice;
v) characterize the immune response against host-adapted borrelia organisms in immunocompetent animals;
vi) illustrate the possibility and risk of the blood transfusion-transmitted infection by *Bp* and *Bb*ss spirochetes.

IV ANIMALS, MATERIALS AND METHODS

1 Experimental design

This study was performed with the following two parts:

i) generation of host-adapted borrelia organisms in immunodeficient mice after ID inoculation of culture-derived motile borrelia spirochetes;
ii) after ID or IV inoculation of the host-adapted spirochetes, observation of the spirochete population dynamics in the immunocompetent mice.

2 Animals

The experimental project was based on murine model and received approval by the government of Upper Bavaria in accordance with § 8 of the German Animal Welfare Act (TierSchG, version of 18 May 2006) (Registration number: AZ 55.2-1-54-2532-144-2015). Mouse strains of immunodeficient NOD-SCID and immunocompetent C3H/HeOuJ (Figure 9) were used for the in vivo experiments of the present dissertation.

Figure 9: Mouse strains used in this study

(a) NOD-SCID mouse; photo from Janvier Labs (Saint Berthevin Cedex, France), **(b)** C3H/HeOuJ mouse; photo from The Jackson Laboratory (Maine, USA)

2.1 Immunodeficient NOD-SCID mouse

In this murine model, immunocompromised mice were firstly used to generate host-adapted borrelia organisms, in order to avoid the interference of any antigen derived from in vitro cultivation. For example, expression of OspA of LB spirochete derived from in vitro cultures differs profoundly from that in arthropod vector and mammalian milieu (DE SILVA & FIKRIG, 1997; CROWLEY & HUBER, 2003). A critical advantage of using the immunodeficient mice is that persistent spirochetemia can be induced, because the lack of immunity allows high bacterial load in the bloodstream compared to that affected by immune responses in immunocompetent animals (HODZIC et al., 2003; LIU et al., 2004; RISTOW et al., 2015). Therefore, host-adapted borreliae can be easily harvested by sampling blood at scheduled time points. The viability and number of spirochetes can be examined under dark field microscope and by PCR method, respectively.

In this study, 35 NOD-SCID (Non Obese Diabetic-Severe Combined Immune Deficiency) mice (Figure 9A), which do not contain mature T and B cells and complement, were purchased at the age of week 6 (~ 15 g body weight) from Janvier Labs at various schedule time. Compared to regular SCID mice, NOD-SCID mice have reduced macrophages and natural kill (NK) cell function (MILSOM et al., 2013).

2.2 Immunocompetent C3H/HeOuJ mouse

Immunocompetent mice develop a well-detectable antibody response in the course of borrelia infection as long as the inoculated spirochetes are infectious. Given that C3H mice may develop severe Lyme arthritis and possess a higher bacterial burden in tissues compared to the resistant strain of BALB/c mice (ZEIDNER et al., 1997; WOOTEN & WEIS, 2001), the immunocompetent C3H/HeOuJ mice (Figure 9B) were used in our study. This mouse strain is easily treatable and tolerated with blood sampling and anaesthesia. At the age of week 8 (~ 20 g body weight), 38 C3H/HeOuJ mice were obtained from Charles River (Sulzfeld, Germany) at different time points based on the experiment scheme.

2.3 Mouse hygiene and handling management

The specific-pathogen-free (SPF) female mice were obtained at different time points based on the work schedule. One to two weeks prior to the experiment mice were introduced into the animal facility of the Institute of Infectious Diseases and Zoonoses, Ludwig-Maximilians-Universität (LMU, Munich) in order to ensure adequate acclimation to the new

environment. According to guidelines and principles of Gesellschaft für Versuchstierkunde/Society of Laboratory Animal Science (GV-SOLAS), maintenance and handling of these mice were conducted in the laminar flow systems to sustain SPF conditions. Three to five mice were kept in an individually ventilated cage (IVC, ISOcage N System; Tecniplast Deutschland GmbH, Hohenpeißenberg, Germany) at controlled room temperature (20 - 24 °C) and humidity (45%). The animals received standard feed for laboratory rodents (housing food V 1536) *ad libitum*; autoclaved water is indefinitely available to the mice from drinking bottles.

Three days prior to the experiment, blood samples were obtained from naïve mice for baseline controls. Collection of blood specimens in small volume (2 - 4 drops ≈ 10 - 30 µl) was carried out from the facial vein of the mouse without anaesthesia. Puncture in the skin was performed with a 4-mm Goldenrod Animal Lancet (Braintree Scientific, Bio-Medical Instruments, Zöllnitz, Germany), which is a rapid and simple method for submandibular bleeding of mice (Figure 10) (GOLDE et al., 2005). The dropping blood from the facial vein was maintained in the Microvette 100 K3E (preparation K_3EDTA; Sarstedt AG & Co., Nümbrecht, Germany). At the end of the experiment, a large final blood volume was harvested aseptically into the S-Monovette 2.7 ml K3E (Sarstedt AG & Co.) by intracardiac puncture. Mice were anaesthetized by IP administration of medetomidine and midazolam (Table 3) (DHEIN et al., 2005; XU, 2006). ID and IV inoculation were conducted on the shaven dorsal back and via the right jugular vein, respectively (procedures in detail will be described in the following sections). Specific antagonists (Table 3) were used to neutralize the anaesthetics. Fentanyl and naloxone provided adequate sedation and analgesia, respectively. Recovery of mice after each challenge were observed closely. The surgical instruments were autoclaved. Standardized aseptic techniques were employed during all procedures. After each experiment based on the scheduled course of period, anaesthetized mice were sacrificed by cervical dislocation.

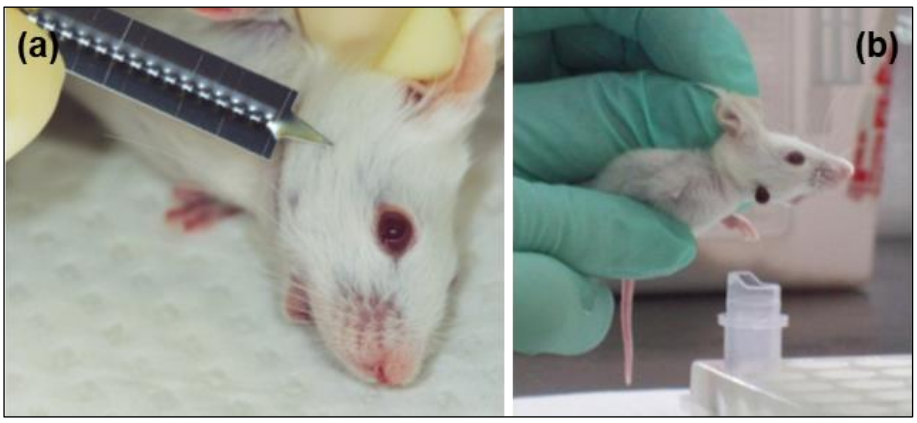

Figure 10: Facial vein bleeding from mouse

(a) positioning and poking the cheek with the lancet; photo from Golde et al. (GOLDE et al., 2005), **(b)** collecting blood from the cheek; photo from Braintree scientific, Inc. (Braintree, USA)

Table 3: Anaesthetics and antagonists used in mice

	drug	dosage (mg/kg)[a]	product unit (mg/ml)	source
anaesthetics	Medetomidine hydrochloride[b]	0.5	1	Dechra Veterinary Products Deutschland GmbH, Aulendorf, Germany
	Midazolam	5	5	Hexal AG, Holzkirchen, Germany
	Fentanyl	0.05	0.05	B. Braun Melsungen AG, Melsungen, Germany
antagonists	Atipamezole	2.5	5	Dechra Veterinary Products Deutschland GmbH
	Flumazenil	0.5	0.1	Hexal AG
	Naloxone	1.2	0.4	B. Braun Melsungen AG

[a] All three substances used as anaesthetics or antagonists were diluted in sterile physiological saline (isotonic saline; B. Braun Melsungen AG); the combined solution was dosed at 10 µl per g body weight of the mouse.
[b] Sedator® 1.0 mg/ml solution

3 Borrelial strains and cultivation

3.1 *Bp* LMU-01 and *Bb*ss N40

In this study, borrelia spirochetes of the following two strains were used to infect the animals:

***Bp* LMU-01 strain** was originally isolated from a heparinized blood specimen of an ill cat in Jerusalem (Israel) (SCHWARZER et al., 2015). After cultivation in filtered human serum, passage 2 of this strain was used with 6.9 x 10^5 *Bp* spirochetes per ml of serum. Aliquots of 100-µl serum were frozen at -80 °C until used. The minimal infectious dose of this borrelia strain in immunocompetent mice has been reported between 4 and 100 organisms per animal (in vitro cultured, passage 3) (SCHWARZER et al., 2016).

***Bb*ss N40 strain** was isolated from a skin biopsy of an experimentally infected dog (APPEL et al., 1993). Its passage 3 was used in a prior study, indicating the infectivity of 1.0 x 10^6 cultured cells in immunocompetent mice (RAFINEJAD et al., 2011). In vitro culture (passage 4) was frozen in the presence of 15% sterile glycerol, containing 1.1 x 10^6 spirochetes in each 100-µl aliquot.

3.2 Preparation of spirochetes for initial inoculation using in vitro culture

Spirochetes used to infect immunodeficient mice were prepared by in vitro cultivation. A list of necessary materials and devices used in this step is given in Table 4. Briefly, a 5-ml polypropylene tube was filled with 3 ml in-house prepared Pettenkofer/LMU Bp medium (SCHWARZER et al., 2015). An aliquot of frozen passage 2 stock (100µl, *Bp* LMU-01 strain) was maintained in this medium at 37 °C with a humidified 5% CO_2 air atmosphere. For cultivation of *Bb*ss spirochetes (at 33 °C), the frozen passage 4 stock (100 µl, *Bb*ss N40 strain) was applied to 6 ml Barbour-Stoenner-Kelly H (BSK-H) complete medium in a 12-ml screw top tube. This medium was additionally supplemented with 6% heat-inactivated (at 56 °C) rabbit serum (POLLACK et al., 1993). The mobility and morphology of the organisms (passage 3 and 5 of *Bp* and *Bb*ss, respectively) were inspected by dark-field microscope. Spirochete counts were achieved in a Petroff-Hausser counting chamber. Viable spirochetes were grown to day 7 and day 6 at the mid-log phase, reaching densities of 2.0 x 10^6 *Bp* and 1.0 x 10^7 *Bb*ss per ml medium, respectively. By adjusting the medium volume to 50 µl and 100 µl, an inoculum dose of 1.0 x 10^5 *Bp* or 1.0 x 10^6 *Bb*ss of motile spirochetes was prepared in the 1.5-ml safe-lock centrifuge tubes for further use. Another inoculation dose of 1.0 x 10^8 viable *Bb*ss organisms was harvested as follows:

i) cultivation of frozen passage 4 stocks of *Bb*ss in 20 of the 12-ml screw top tubes for 6 days to the concentration of ~ 1.0 x 10^7 cells/ml as described above;
ii) centrifugation of 120 ml culture in 3 of the 45-ml centrifuge tubes (each 40 ml) at 15,000 × g for 10 min at 23 °C;
iii) washing the three pellets with 1 ml sterile phosphate-buffered saline (PBS, pH 7.3);
iv) transfer of 3 ml PBS into a 5-ml sterile safe-lock centrifuge tube;
v) centrifugation of 5-ml tube at 15,000 × g for 10 min at 23 °C;
vi) resuspension of the pellet in 1 ml fresh BSK-H medium;
vii) dilution of 10-µl re-suspended solution (1:100) and count for the concentration of live spirochetes (1.0 x 10^9 cells/ml);
viii) adjustment with appropriate medium volume (100 µl) for further use.

Table 4: Materials used for in vitro cultivation and preparation of inoculum dose

instrument, ingredient	comment	source
BSK-H complete medium		Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
centrifuge 5430R and 5810R		Eppendorf Vertrieb Deutschland GmbH, Wesseling-Berzdorf, Germany
centrifuge tube	45-ml	Sarstedt AG & Co.
dark-field microscope		Leica Microsystems GmbH, Wetzlar, Germany
Galaxy 170S CO_2 incubator		Eppendorf Vertrieb Deutschland GmbH
Petroff-Hausser counting chamber		Hausser Scientific, Horsham, PA, USA
polypropylene tube	5-ml	Sarstedt AG & Co.
rabbit serum	activated	Sigma-Aldrich Chemie GmbH
safe-lock centrifuge tube	1.5-ml, 5-ml	Eppendorf Vertrieb Deutschland GmbH
screw top tube (centrifuge tube 12)	12-ml, TPP	Faust Lab Science GmbH, Klettgau, Germany

4 Experimental procedures

4.1 Negative control group

Before the experiments that animals were exposed to spirochetes, a negative group (NG) was performed as control (Table 5). Briefly, anticoagulated whole blood (free of borrelia organisms) were collected from two non-infected NOD-SCID mice. One hundred microliter blood was IV injected into five immunocompetent C3H/HeOuJ mice under anaesthesia. The challenged mice were monitored for five weeks; blood was collected once a week. Once ensured that there was no inflammation on the surgical area (Figure 11) and no microsurgery-induced specific antibody levels, the experimental test with borrelia organisms was then performed.

Table 5: Mice used for negative control

mouse	strain	subgroup	procedure	number of animals
negative group (NG)	NOD-SCID	N-NG	Harvesting of blood	2
	C3H/HeOuJ	C-NG	IV inoculation	5

Figure 11: Recovery of C3H/HeOuJ mouse after microsurgery via the jugular vein

(a) recovery of sutured wound on day 2 after microsurgery, **(b)** recovery of sutured wound on day 6 after microsurgery

4.2 Generation of host-adapted borreliae in NOD-SCID mice

4.2.1 Intradermal inoculation of culture-derived spirochetes

As described above, the inoculum dose of 1.0 x 10^5 *Bp* or 1.0 x 10^6 (or 1.0 x 10^8) *Bb*ss spirochetes was freshly prepared before use. Each of six NOD-SCID mice was ID injected with the borrelial organisms (*Bp* or *Bb*ss) into the shaven back (Figure 12). The time point was named day 0. The injection volume was divided into small portions (5 x 10 µl of *Bp*, 10 x 10 µl of *Bb*ss), placed closely to each other into the dermis (~ 4 cm^2 area). Thereafter, six mice were randomly separated into two subgroups (3 mice of each) (Table 6).

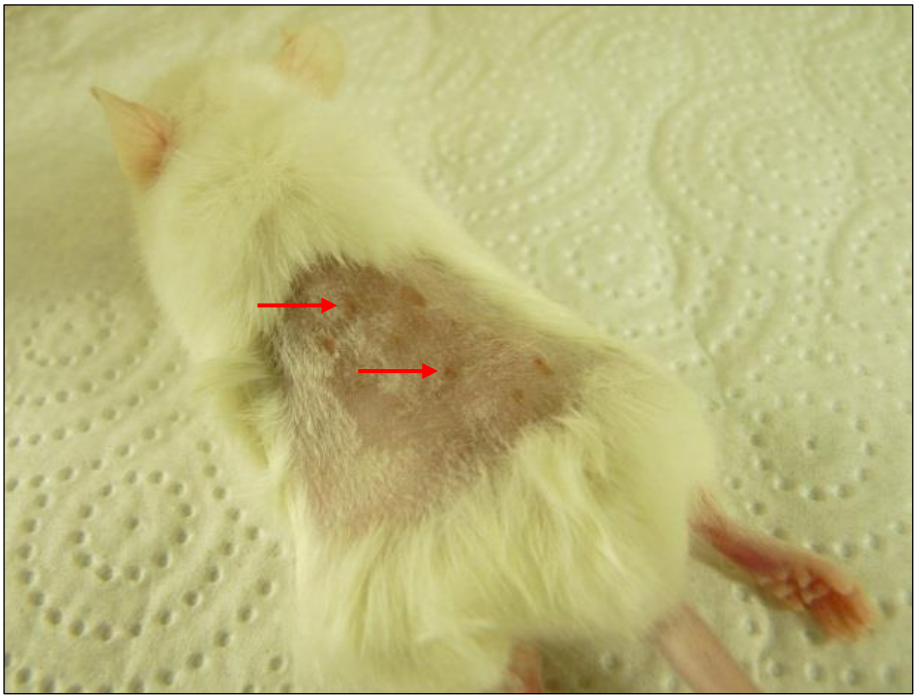

Figure 12: Intradermal injection of culture-derived spirochetes into the shave back of NOD-SCID mouse under anesthesia

Table 6: NOD-SCID mice used to detect populations of host-adapted borreliae

borrelial species	injection route	inoculum dose	subgroup	number
Bp (passage 3)	ID	1.0 x 10^5	N-A	3
			N-B	3
*Bb*ss (passage 5)	ID	1.0 x 10^6	N-C	3
			N-D	3
		1.0 x 10^8	N-E	3
			N-F	3

4.2.2 Dynamic detection of host-adapted borreliae in blood

In order to obtain data for each single day of the scheduled 17-day interval, mice in different subgroups were bled via facial vein puncture according to the alternating scheme. Briefly, blood specimens (~ 10 - 30 µl) from each single mouse in subgroup N-A, N-C, and N-E were collected at odd days (day 1, 3…17) and subgroup N-B, N-D, and N-F at even days (day 2, 4…16) (Figure 13). Puncture alternated between the right and left facial vein at each sampling day to produce less impact on the mice as possible. Blood aliquots (5 µl each) from each single mouse at each time point were separated in individual 1.5-ml safe-lock tubes and stored at -30 °C. By DNA extraction and PCR examination, the population kinetics of the hematogenous spirochete load from six NOD-SCID mice that received the same inoculum dose was monitored between day 0 and 17. The term "the anticipated day", namely the time point to reach the largest bacteria load in the blood was determined during this step. Additionally, 30-µl blood from three mice in each individual subgroup was diluted and mixed with 500 µl medium for *Bp* or *Bb*ss cultivation, followed by an examination for motile spirochetes using dark-field microscope. Once no viable spirochete was observed, the solution above was transferred to 5 ml corresponding medium for further growth and inspection of potential *Bp* or *Bb*ss spirochetes. The ~ 5.5-ml culture medium used here contained antibiotics (Table 7).

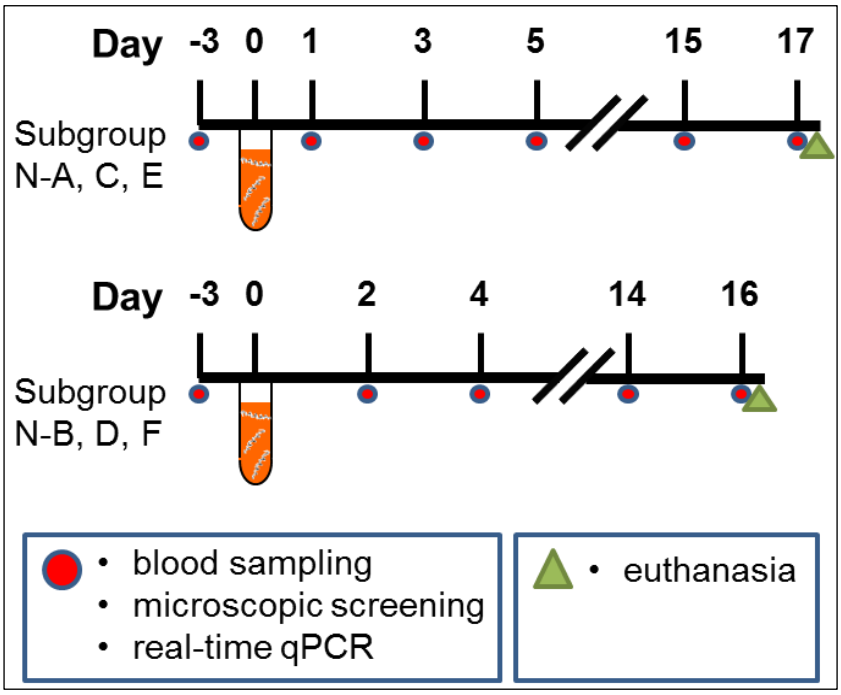

Figure 13: Blood sampling from NOD-SCID mice based on alternative schedule

Table 7: Antibiotics used in medium for spirochete isolation from blood samples

antibiotic	product No.	concentration	source
Kanamycin[a]	K0129	8 µg/ml	Sigma-Aldrich Chemie GmbH
Rifampicin[b]	R3501	50 µg/ml	

[a] 20 ml solution, 10 mg kanamycin per ml 0.9% sterile NaCl
[b] 250 mg powder stock, dissolved in 5 ml DMSO (dimethyl sulfoxide, Sigma-Aldrich Chemie GmbH)

4.2.3 Harvesting of host-adapted borreliae in blood

Based on the obtained population kinetics of spirochetes circulating in the bloodstream, a second separate group consisting of five NOD-SCID mice was performed using ID inoculation of the in-medium cultured 1.0 x 10^5 *Bp* or 1.0 x 10^6 *Bb*ss spirochetes (Table 8). Blood samples at a time point before the anticipated day were collected to determine if these mice were infected with borreliae. At the anticipated day with the highest spirochete load, anticoagulated peripheral blood was collected aseptically by intracardiac puncture under anaesthesia. A S-Monovette 2.7 ml K3E was connected with a 23-gauge needle for puncture into the heart and utilized for the maintenance of the anticoagulated blood (~ 0.8 - 1.2 ml) from each individual mouse (Figure 14). Thirty microliter blood from each single mouse were diluted in 500 µl fresh medium and motile spirochetes were visualized with a dark-field microscope. All blood samples containing *Bp* or *Bb*ss spirochetes were pooled in one S-Monovette 2.7 ml K3E and checked again for bacterial viability with the dark-field microscope. Aliquots (5 µl) from pooled blood were prepared and stored at -30 °C for borrelial density determination.

Table 8: NOD-SCID mice used for generation of host-adapted borrelia organisms

borrelial species	injection route	inoculum dose	subgroup	number	mouse no.
Bp (passage 3)	ID	1.0 x 10^5	N-G	5	N1-5
*Bb*ss (passage 5)	ID	1.0 x 10^6	N-H	5	N6-10
			N-I	5	N11-15

Figure 14: Blood collection by intracardiac puncture

(a) technique model of blood collection by intracardiac puncture; photo from Howard University (HOWARD UNIVERSITY), **(b)** collected anticoagulated blood in S-Monovette 2.7 ml K3E

4.2.4 Collection of tissue samples for cultivation and PCR testing

Besides blood, other tissues including ear, skin (injection areal), tibiotarsal joint, spleen, urinary bladder, heart, and brain were removed from the sacrificed NOD-SCID mice (subgroup N-G and N-H) at the anticipated day (protocol seen in the following section). Each collected tissue sample was divided into approx. equal two parts. Half of each tissue sample was transferred to medium for bacterial culture and the other half was kept at -80 °C for DNA extraction and further PCR testing.

4.3 Inoculation of host-adapted borreliae into C3H/HeOuJ mice

4.3.1 Intradermal inoculation of host-adapted borreliae

Anaesthetized immunocompetent C3H/HeOuJ mice (n=16; Table 9) were intradermally inoculated with 100 µl freshly pooled blood containing the NOD-SCID-adapted borrelia organisms (*Bp* or *Bb*ss) in the shaven dorsal back (the same ID injection technique as used for NOD-SCID mice).

Table 9: Subgroups of C3H/HeOuJ mice used for ID and IV inoculation

borrelial species[a]	NOD-SCID	C3H/HeOuJ			
	subgroup	subgroup	injection route	number	mouse no.
Bp	N-G	C-A	ID	5	C1-5
		C-B	IV	6	C6-11
*Bb*ss	N-H	C-C	ID	5	C21-25
		C-D	IV	5	C26-30
	N-I	C-E	ID	6	C31-36
		C-F	IV	6	C37-42

[a] Borrelial organisms were host-adapted and present in pooled blood from NOD-SCID mice in each subgroup.

4.3.2 Strict intravenous inoculation of host-adapted borreliae

C3H/HeOuJ mice (n=17) were strictly IV inoculated with the same pooled blood as used in each subgroup of ID injection (Table 9). Each mouse was transferred into the open space of the SPF-conditioned animal handling room (Figure 15). The IV inoculation was carried out via the right jugular vein (Figure 16) and instruments used in this procedure are listed in Table 10. Briefly, C3H/HeOuJ mouse was deeply anaesthetized for the surgical procedure. The warming pad was used to maintain the body temperature at ~ 37 °C. Hair under the chin was shaved and this area was disinfected with 70% ethanol. On the position slightly right to the body midline under the chin, a ~ 1 cm long incision was made into the skin. Using the stereo microscope equipped with Leica MC170 HD camera and two goose lights (Figure 15), the right jugular vein was carefully separated from the connective tissue and fat without any bleeding. Splinter forceps and eye forceps were utilized during this procedure. The exposed vein and removed tissue were immersed in sterile physiological saline. By holding the jugular vein with bulb-headed probe, two loop ligations were made around the vein with SURGICRYL® PGA Polyglycolic acid suture. The up-ligation was completely closed and the down-ligation was a loose knot fixed with Student Halsted-Mosquito Hemostats. The jugular vein was held well with two bulb-headed probes to avoid bleeding when a 45°-angle hole on the up side of the vein was cut by the Vannas-style spring scissors. The micro hook assisted greatly to open and grab the wall of the vessel for inserting the catheter. When ~ 9 mm of the Alzet Mouse Jugular Catheter (filled with ~ 13 µl sterile isotonic saline) tip was introduced parallel into the jugular vein, the down-ligation was closed and secured that

the catheter would not slip out from the vein. The down-put bulb-headed probe was removed from the jugular vein for adequate space and fluent injection. After connecting the Terumo Agani needle (attached to the 1-ml syringe filled with 140 µl pooled blood) with the exposed end of the catheter, 100 µl blood were injected very slowly (~ 10 µl/min) into the vein. Thereafter, the needle was removed from the catheter and a second 23-gauge needle connected with a 1-ml syringe (filled with sterile saline) was subsequently attached to the catheter. Then, the catheter was flushed slowly with 50 µl sterile saline. Without withdrawing the needle and syringe the catheter tip was carefully pulled back. Simultaneously and immediately, the down-ligation was fastened entirely without any bleeding into the surrounded tissue. After the injection, the incision site was sutured intracutaneously (4 - 5 single sutures) with Monosyn® Easyslide. Then, antagonists were injected intraperitoneally for recovery. The procedure, from making the incision on the chin until closing the skin, took approx. 60 minutes per mouse. During the IV inoculation, movie (available on the CD-ROM) was taken through 10x/1.6 objectives on the stereo microscope.

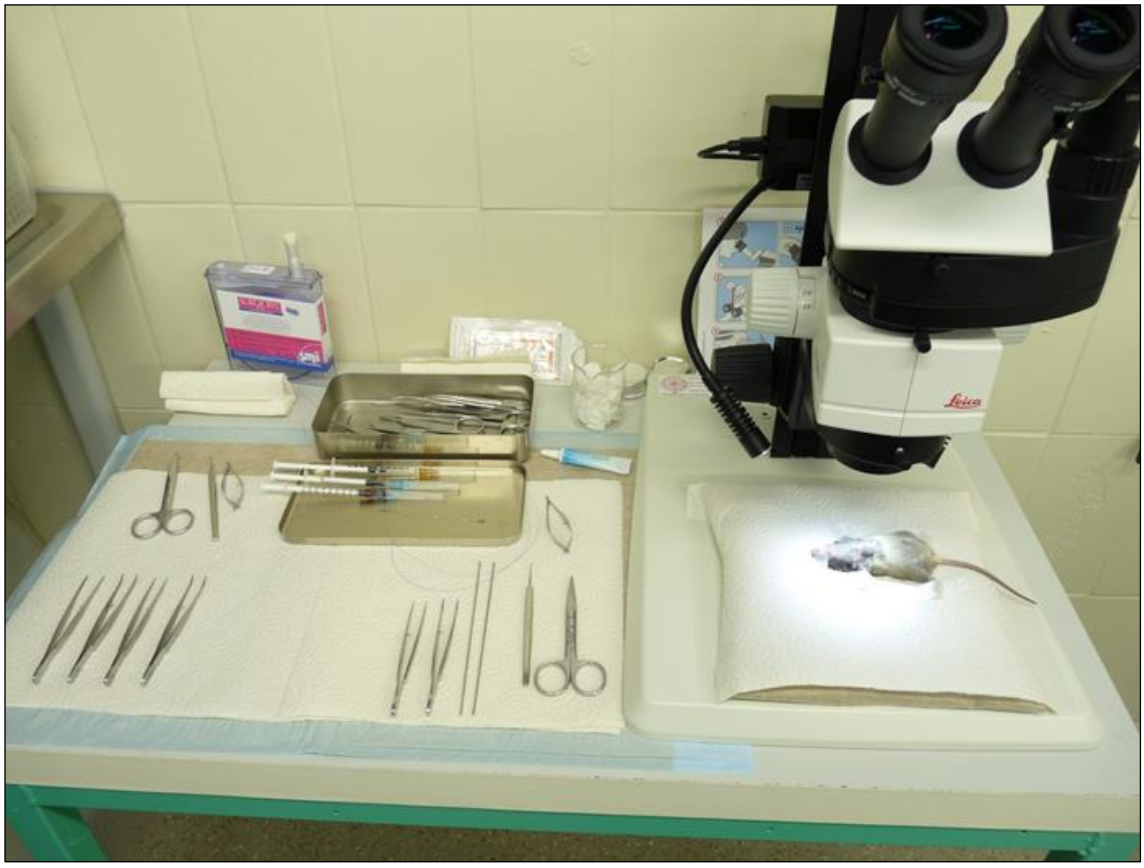

Figure 15: Working condition and anaesthetized mouse before IV inoculation

mouse kept on a warming pad

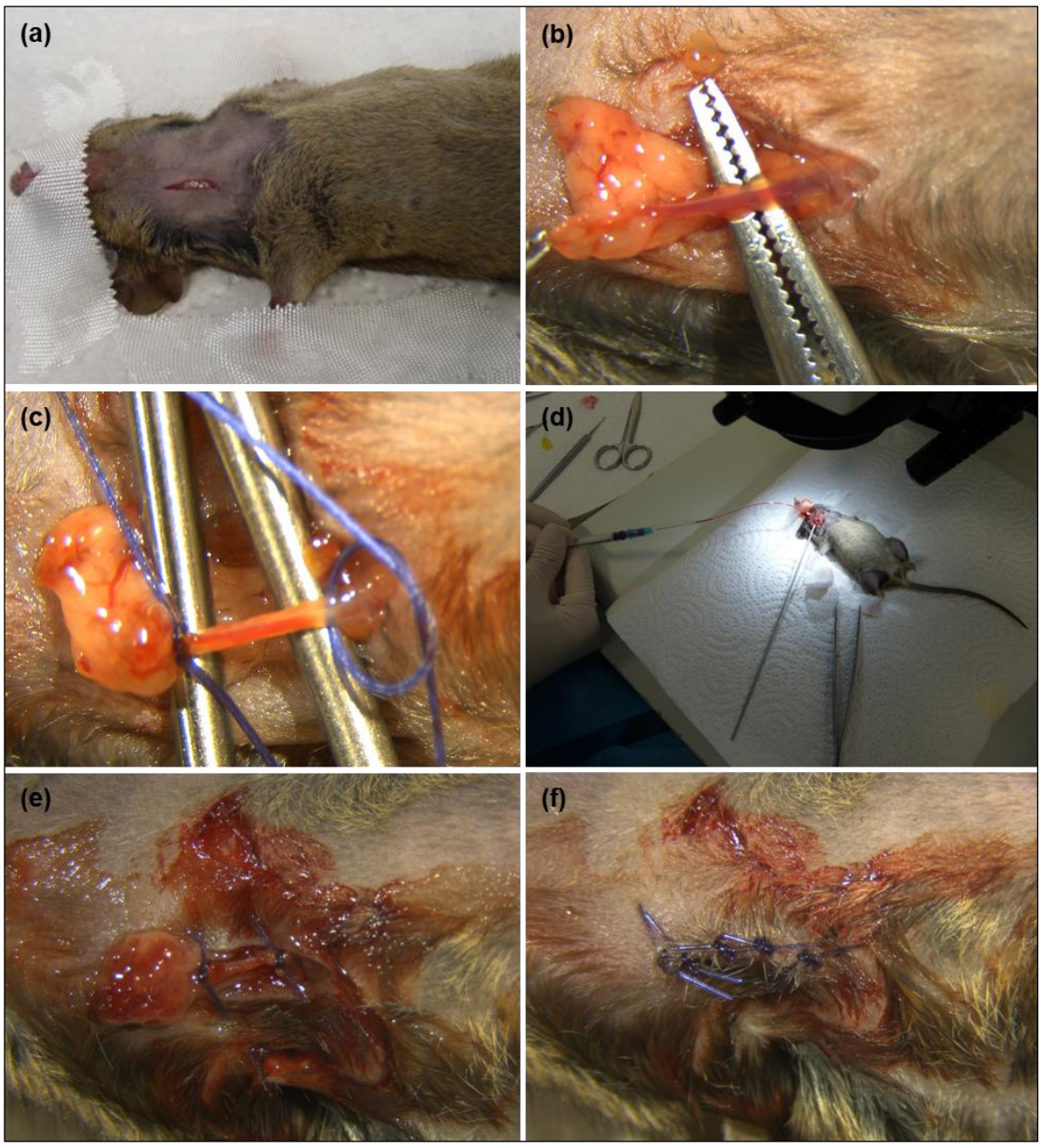

Figure 16: Strict IV injection via the jugular vein monitored with a stereo microscope

(a) a 1-cm long incision in the skin under the chin, **(b)** right jugular vein separated from connective tissue and fat, **(c)** up-ligation (fixed) and down-ligation (a loose knot), **(d)** injection of pooled blood containing host-adapted borreliae into right jugular vein, **(e)** two ligations around the right jugular vein after injection, **(f)** closed skin after injection

Table 10: Materials used for microsurgery via the jugular vein

instrument	comment	source
Alzet Mouse Jugular Catheter	No. 0007700	Durect Corporation, California, USA
bulb-headed probe	No. 310335	Henry Schein VET GmbH, Hamburg, Germany
camera	Leica MC170 HD	Leica Microsystems GmbH
eye forceps	No. 310174	Henry Schein VET GmbH
goose light	Leica LED3000 SLI	Leica Microsystems GmbH
micro hook	No. 10062-12; blunt	Fine Science Tools GmbH, Heidelberg, Germany
Monosyn® Easyslide suture	DS16 (5/0)	B. Braun Surgical SA, Barcelona, Spain
single use syringe	1-ml	Dispomed Witt oHG, Gelnhausen, Germany
splinter forceps	No. 310645	Henry Schein VET GmbH
stereo microscope	Leica M60	Leica Microsystems GmbH
Student Halsted-mosquito hemostats	No. 91309-12	Fine Science Tools GmbH
SURGICRYL® PGA Polyglycolic acid suture		SMI AG, St. Vith, Belgium
Terumo Agani needle	23-gauge	Shanghai international Holding Corporation GmbH (Europe), Hamburg, Germany
Vannas-style spring scissors	No. 15000-03	Fine Science Tools GmbH
warming pad	Model #39DP	Braintree scientific, Inc., Braintree, USA

4.3.3 Blood collection, plasma and serum samples

After the ID and IV injection, blood samples were collected via facial vein bleeding from C3H/HeOuJ mice at indicated intervals (Figure 17). Considering the recovery time of the surgical skin of the IV inoculated mice (Figure 11), the first blood samples were collected

on day 6 after surgery and subsequently every three days until day 30 post inoculation (p.i.). Afterwards, bleeding was performed on day 37, day 44, and day 49/50 (ID challenged mice on day 49, IV mice on day 50). Aliquots of blood (5 µl) were prepared from each single mouse at each time point. Plasma was obtained from pooled blood from each ID or IV subgroup by soft centrifugation at 350 × g for 10 min at 23 °C. On day 49/50, final blood samples via intracardiac puncture were collected with a 23-gauge needle connected with a 2-ml sterile single use syringe (Henry Schein VET GmbH). Non-coagulated blood was kept in a micro tube (1.1 ml Z-Gel; Sarstedt AG & Co.) and serum sample was harvested by centrifugation as describe above. All samples were stored in the 1.5-ml safe-lock tubes at -30 °C for subsequent tests.

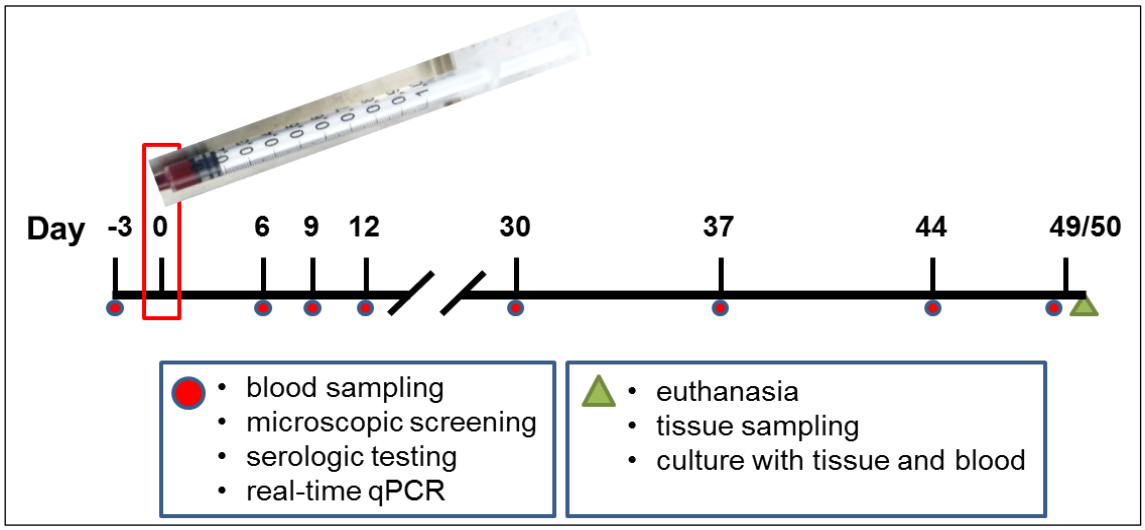

Figure 17: Blood sampling from C3H/HeOuJ mice

4.3.4 Culture of spirochetes from blood and tissue samples

Thirty microliter pooled blood from C3H/HeOuJ mice of ID or IV challenged subgroup were diluted and mixed with 500 µl culture medium (for *Bp* or *Bb*ss cultivation). Detection of motile spirochetes was performed using dark-field microscope. Once no viable spirochetes were observed, the solution above was transferred to 5 ml medium for culture of *Bp* or *Bb*ss spirochetes. The ~ 5.5-ml culture medium used was supplemented with kanamycin and rifampicin (Table 7) to supress undesirable contaminating bacterial growth.

On day 49/50 p.i., various tissue samples from the ear, skin (injection areal; ~ 6 mm diameter), tibiotarsal joint, spleen, urinary bladder, heart, and brain were collected from each

euthanatized C3H/HeOuJ mouse. Tissues were immersed in 70% ethanol for 1 min and then washed with sterile PBS (pH 7.3) for 30 seconds. Ear and skin tissues were immersed in 70% ethanol for 2 min. After washing, tissues were cut into approx. equal two parts. Half of each sample was put in a 1.5-ml microcentrifuge tube and stored at -30 °C for DNA extraction and further PCR testing. For spirochete cultivation the other half part was transferred into a sterile Stomacher closure bag (Seward Laboratory, London, UK), which contained 2 ml Pettenkofer/LMU *Bp* medium (for *Bp*) or 2 ml BSK-H complete medium (for *Bb*ss) devoid of any antimicrobials. Once tissue samples from all mice in ID and IV subgroups were obtained, the Stomacher bags were processed at normal speed for 60 seconds with a Stomacher® 80 *micro*Biomaster (Seward Laboratory). The pressed tissue sample and medium was subsequently transferred into the 12-ml screw top tube prefilled with 8 ml of the same medium. All cultures were kept at 37 °C (*Bp*) or 33 °C (*Bb*ss) for six weeks and inspected once a week using a dark-field microscope.

4.4 Detection and quantification of borrelial DNA in blood and tissue samples

4.4.1 DNA extraction

All blood and tissue samples from the NOD-SCID and C3H/HeOuJ mice were subjected to DNA extraction using AS3000 Maxwell 16 MDx Instrument and the Maxwell 16 LEV Blood DNA Kit. Reagents and materials used in this experiment are listed in Table 11. The protocols, which were successfully applied in a previous study (SCHWARZER et al., 2016), are depicted in Figure 18. The extracted DNA was eluted in 50 µl elution buffer and frozen in the 1.5-ml safe-lock tube at -30 °C until used.

Table 11: Materials and reagents used for DNA extraction

name	source
1.5-ml safe-lock tube	Eppendorf Vertrieb Deutschland GmbH
AS3000 Maxwell 16 MDx Instrument	Promega GmbH, Mannheim, Germany
incubation buffer	Promega GmbH
Maxwell 16 LEV Blood DNA Kit[a]	Promega GmbH
micro pestle	Faust Lab Science GmbH
PBS	in-house made, sterile, pH 7.3
ThermoMixer comfort 5355 V 2.0	Eppendorf Vertrieb Deutschland GmbH

[a] containing lysis buffer, proteinase K, and elution buffer

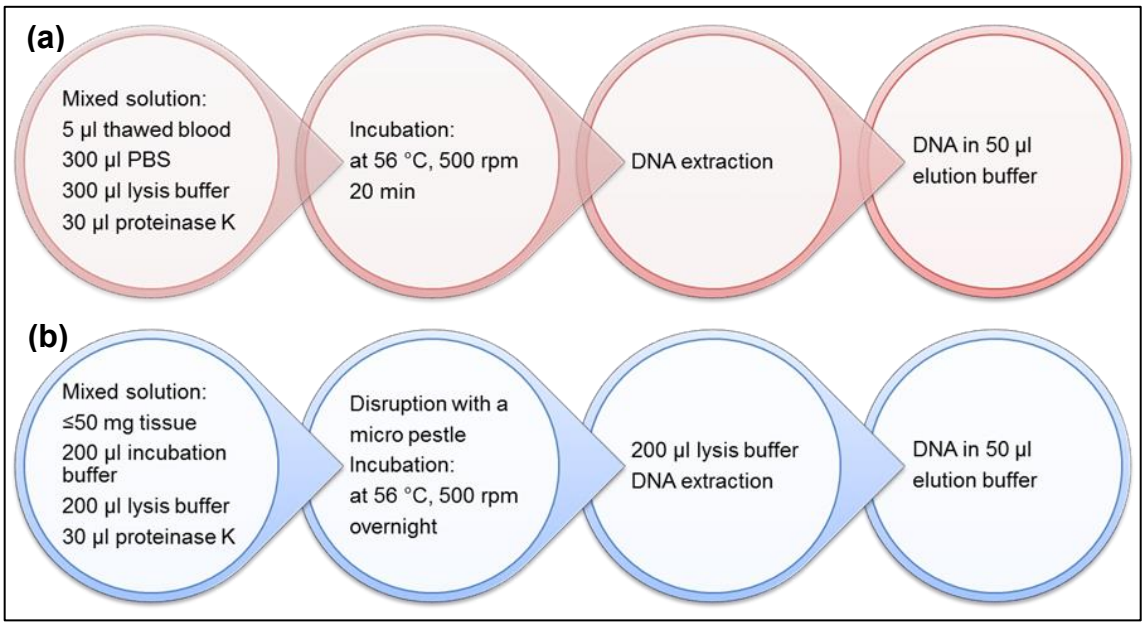

Figure 18: Borrelial DNA extraction from blood (a) and tissue samples (b)

4.4.2 PCR testing

Borrelia-specific DNA was detected and quantified using the QuantStudio 5 real-time quantitative PCR (qPCR) system (Applied Biosystems, ThermoFisher Scientific GmbH, Ulm, Germany). The target sequence of *flaB* (*Bp*) and *ospA* (*Bb*ss) genes (Table 12) have been described in the prior studies (STRAUBINGER, 2000; SCHWARZER et al., 2016). Oligonucleotide primers and probes of the genes were synthesized by Eurofins Genomics (Ebersberg, Germany). Each qPCR reaction was set up in a 20 µl final volume (Table 13). The qPCR running program consisted of (i) heating at 95 °C for 2 min for polymerase activation and DNA denaturation, (ii) amplification for 40 cycles with denaturation at 95 °C for 5 s and extension and annealing at 60 °C for 25 s, (iii) a final step at 25 °C for 2 min.

Table 12: Primers and probes used in this study

primer name	sequence
*Bp_flaB*_fw	5'-GAGGGTGCTCAACAAGCAA-3'
*Bp_flaB*_re	5'-CAACAGCAGTTGTAACATTAACTGG-3'
*Bp_flaB*_probe	5'-FAM-AAATCAGGAAGGAGTACAACCAGCAGCA-3'-TAM
*Bb*ssN40-*ospA* 17 fw	5'-AATGTTAGCAGCCTTGACGAGAA-3'
*Bb*ssN40-*ospA* 119 re	5'-GATCGTACTTGCCGTCTTTGTTT-3'
*Bb*ssN40-*ospA*-41T	5'-FAM-AACAGCGTTTCAGTAGATTTGCCTGGTGA-3'-TAM

Table 13: PCR reaction used in this study

PCR	*Bp* (*flaB*)		*Bb*ss (*ospA*)	
	working concentration	reaction volume (µl)	working concentration	reaction volume (µl)
master mix[a]	1x	10	1x	10
forward primer	600 nM	1.2	900 nM	0.64
reverse primer	600 nM	1.2	900 nM	0.64
probe	200 nM	0.8	100 nM	0.97
reference dye[b]		0.1	-	-
nuclease-free water		4.2		5.25
template DNA		2.5		2.5

[a] QuantiNova Probe PCR Master Mix (Qiagen GmbH, Hilden, Germany)
[b] QN ROX reference dye (Qiagen GmbH)

In order to quantify spirochetes in the DNA samples extracted from blood or tissue samples, a PCR-based standard curve was established. Briefly, double stranded DNA (dsDNA) targeting the sequence of *flaB* (*Bp*) or *ospA* (*Bb*ss) genes with known quantities (0.5 nmol) was synthesized by Metabion International AG (Planegg, Germany). The dry dsDNA stock was diluted in 5 µl nuclease-free water (0.1 nmol/µl). Considering that 1.0 mol is 6.022×10^{23} molecules, concentration of the target gene copy numbers in this dsDNA solution was 6.022×10^{13} molecules/µl. Ten-fold serial dilutions were prepared with nuclease-free water. Dilutions ranging from 6.022×10^{7} to 6.022×10^{0} molecules/µl were used to run a qPCR procedure as described above. After analysis using the QuantStudio Design & Analysis Software (Applied Biosystems), the standard curve was established, which was determined by the threshold cycles (Ct) of the dsDNA copy numbers at each dilution. Ten-microliter aliquots of dsDNA solution at concentration of 6.022×10^{5} and 6.022×10^{3} molecules/µl, were prepared and stored at -30 °C before use.

In each run of the qPCR system a no-template-control (NTC, 2.5 µl nuclease-free water) and two positive controls of dsDNA (6.022×10^{5} and 6.022×10^{3} molecules/µl as prepared above) were included in the 96 Multiply PCR plate natural (Sarstedt AG & Co.). Both standard template dsDNA and sample DNA from blood and tissue samples were amplified in triplicate. Spirochete number per ml blood or per mg tissue were obtained with the QuantStudio Design & Analysis Software (Applied Biosystems) considering Ct-values for each sample analysed.

4.5 Serological analysis

4.5.1 ELISA

Antigen production was prepared from cultured spirochetes of *Bp* and *Bb*ss. The purified borrelial cell lysate was used to detect mouse antibodies by ELISA test. Spirochetes of the frozen stocks of *Bp* LMU-01 (passage 2) and *Bb*ss N40 (passage 4) were grown in medium as described in section IV.3 (borrelial strains and cultivation). When reaching the desired concentration of 10^7 to 10^8 spirochetes/ml, the 6-ml bacteria suspensions of *Bp* or *Bb*ss were transferred to a sterile airtight screw cap glass bottle containing 1.0 l of the corresponding medium. The cultures were further incubated for 7 to 9 days to stationary phase. According to the protocols described by earlier studies (TÖPFER, 2005; TÖPFER & STRAUBINGER, 2007), one liter of the culture was separated into 250- ml tubes and centrifuged at 10,000 × g at 20 °C for 15 min. After discarding the supernatants, the pellets were re-suspended in sterile PBS. The suspensions were centrifuged at 10,000 × g at 4 °C for 15 min. This step was repeated twice. After the last centrifugation, the spirochetes were re-suspended in 5 ml sterile PBS and placed on ice. Ultrasound disruption of these borrelia organisms was done on ice at 35 W for 3 to 5 s, repeating the procedure for 3 times (Bandelin Sonoplus UW2070, Berlin, Germany). After controlling the bacteria disintegration applying dark-field microscope, the solution of disrupted cells was centrifuged at 10,000 × g at 4 °C for 10 min. The supernatant of the whole-cell lysate was collected and stored at -30 °C until used. Protein concentration of the *Bp* or *Bb*ss cell lysate was measured using the bicinchoninic acid (BCA) protocol (BCA Protein Assay Kits; Pierce Biotechnology Inc., Rockford, USA). The quality of the antigen preparation was determined using SDS-PAGE gels stained with silver nitrate salt (SilverQuest Silver Staining Kit; Invitrogen GmbH, Karlsruhe, Germany).

ELISA plates were prepared by coating the whole-cell antigen lysate of *Bp* and *Bb*ss in the microdilution plates (Nunc-Immuno Microwell Maxisorp C96; Thermo Scientific, VWR International GmbH, Ismaning, Germany). According to the published protocol (BARTH et al., 2014; SCHWARZER et al., 2016), a solution of carbonate coating buffer (CO3, 0.1M), CO3/ME/SDS, and borrelia antigen was prepared. Preparation of CO3 and CO3/ME/SDS were described by Töpfer (TÖPFER, 2005). The final concentration of *Bp* or *Bb*ss antigen proteins was 0.2 µg (*Bp*) or 0.18 µg (*Bb*ss) per 100 µl of each well, respectively. For the *Bb*ss-ELISA plate, pure recombinant OspA (1 ml/1 dose, Rekombitek Lyme; Merial, Duluth, GA, USA) was also applied in the solution at a concentration of 0.32 µl per well (100 µl). Half of the wells received 100 µl aliquots of the prepared antigen solution and the remaining

wells were coated with only 100 µl of CO3, serving as negative control values. Coated plates were covered and incubated at 4 °C overnight. Then, the plates were stored at -30 °C over a 24-hour period until used.

A computerized kinetic ELISA (KELA) test was carried out to detect the specific antibodies against *Bp* or *Bb*ss. Before use, the coated 96-well ELISA plates and the collected plasma and serum samples from inoculated C3H/HeOuJ mice were thawed to room temperature. Each test included three negative and three positive controls. Negative plasma samples derived from borrelia-free C3H/HeOuJ mice (in the negative control group see section IV.4.1) and positive serum samples were from previously *Bp* or *Bb*ss infected mice. All plasma and serum samples were tested in duplicate and mean values are reported. The washing process for the 96-well plate was done with an AquaMax® microplate washer (Molecular Devices, Sunnyvale, USA). All subsequent washes were performed with washing buffer of PBS containing 0.05% Tween 20 (AppliChem GmbH, Darmstadt, Germany). Samples of plasma and serum were diluted at 1:100 with sample buffer PBS containing 0.05% of Tween 20 and 2% non-fat dry milk (Merck KGaA, Darmstadt, Germany). After one wash with washing buffer, 100 µl of the diluted samples were added to each well (in duplicate each with lysate antigen and carbonate buffer control) and incubated for 1 h at 37 °C. After a second wash, 100 µl of 1:4000 (for *Bp*) or 1:3000 (for *Bb*ss) diluted secondary peroxidase-conjugated goat anti-mouse immunoglobulins (IgG, IgA, IgM; MP Biomedicals, LLC, Heidelberg, Germany) were added to each well and incubated for 30 min at room temperature. After a final wash, 100 µl of the substrate (TMB Microwell Peroxidase Substrate Kit; KPL, medac GmbH, Wedel, Germany) were immediately added to each well (within 1 min 45 s). The extinction of each well was immediately read 5 times at 650 nm in 35-s intervals in a SpectraMax Plus 384 Microplate Reader (Molecular Devices, Wokingham, UK). Results were analyzed with the SoftMax Pro software 5.3 (Molecular Devices). To standardize the sample assessment and compare the plates of every run, all results of the tested specimens in antibody units were adjusted to the evaluated values of the control samples. Note that the same control samples were performed for each ELISA test in the same *Bp* or *Bb*ss experiments.

4.5.2 LIA

Serum samples collected from C3H/HeOuJ mice on day 49/50 p.i. with *Bb*ss were analysed with a specific line immunoblot assay (LIA), in order to visualize specific antibodies against

this agent. Briefly, the commercially available IgG immunoblotting strips (Sekisui Virotech GmbH, Rüsselsheim, Germany) were used according to the manufacturer's instructions. The recombinant protein fractions of VlsE mix, OspA mix, DbpA mix, OspC mix, BmpA, p58, and p83/100 antigens (Table 14) are considered to be specific for LB infection in humans and animals (BRUCKBAUER et al., 1992; ZÖLLER et al., 1993). Serum was 1:100 diluted in the ready to use IgG immunoblot dilution-/wash buffer (Sekisui Virotech GmbH). At a dilution of 1:1,000, secondary peroxidase-conjugated goat anti-mouse immunoglobulins (IgG, IgA, IgM; MP Biomedicals) were incubated with the strips for 30 min at room temperature. After three washing steps with dilution-/wash buffer and one time with distilled water, the colour reaction was initiated by adding substrate solution (Opti-4CN Substrate Kit; Bio-Rad Laboratories GmbH) and stopped by washing the strips 3 times with distilled water.

Table 14: Name and molecular weight of *Bb*ss antigens used for LIA test

name	molecular weight (kDa)	origin/function	reference
DbpA	17-18	Osp, immunodominant, chronic infection	JAURIS-HEIPKE et al., 1999
OspC	22-24	Osp, early infection (see section II.3.2)	PAL et al., 2004
OspA	31-33	Osp, early and late infection (see section II.3.2)	WILSKE et al., 1993
BmpA	39	Osp, immunodominant, role in biology of borrelia	SHIN et al., 2004
p58	58	Osp, immunodominant	HAUSER et al., 1997
VlsE	66	in vivo-expressed protein, plasmid encoded, persistent infection (see section II.3.5.2)	BYKOWSKI et al., 2006
p83/100	83-100	protein from protoplasmic cylinder, highly specific for chronic infection	BRUCKBAUER et al., 1992

4.6 Statistics and data analysis

Bacteria-free blood and tissue samples, which were collected from mice before borreliae exposure, served as negative controls. All data for graphs in this study were prepared with the OriginPro 2017 Software (Additive GmbH, Friedrichsdorf, Germany). Data obtained from each single mouse in the same subgroup are presented as mean and standard deviation (SD).

The students unpaired, two-tailed *t* test was utilized to determine if there is a significant difference between mouse subgroups after ID or IV inoculation of *Bb*ss spirochetes. Difference was considered statistically significant when this test resulted in *p* values that were lower than 0.05.

V RESULTS

1 Population kinetics of host-adapted borreliae in the blood of immunodeficient NOD-SCID mice

Six immunodeficient NOD-SCID mice were ID challenged with an inoculum dose of in vitro cultured 1.0 x 10^5 *Bp* or 1.0 x 10^6 (or 1.0 x 10^8) *Bb*ss spirochetes. During a 17-day infection course, viable borrelia organisms and their numbers in the bloodstream were recorded.

1.1 Kinetics of host-adapted *Bp* spirochetes

On day 1 after ID inoculation, *Bp* DNA targeting *flaB* gene (1.3 ± 0.2 x 10^5 copies/ml) was detectable in the blood samples from six mice in subgroup N-A and N-B by qPCR testing (Figure 19). Viable spirochetes were also observed in the blood samples after dilution (30 µl blood in 500 µl medium). Borrelial counts increased the following days after inoculation. Although, minor drop-offs occurred on day 4 (2.2 ± 1.7 x 10^5 cells/ml), day 7 (1.8 ± 0.5 x 10^6 cells/ml), and day 11 (3.6 ± 2.0 x 10^6 cells/ml) (Figure 19). Considering the number and viability of *Bp* occurring between day 1 and day 17 (8.8 ± 1.2 x 10^6 cells/ml) in the bloodstream, day 12 (5.6 ± 1.2 x 10^6 cells/ml blood) was determined as the anticipated time point to obtain the blood samples for further experiments. Subsequently, each of the other five NOD-SCID mice (subgroup N-G) were ID inoculated with cultured 1.0 x 10^5 *Bp* organisms. On day 6 p.i., each microliter blood was detected with 1.5 ± 0.1 x 10^6 spirochetes. On day 12, 7.2 x 10^6 *Bp* organisms were detected in one millilitre of the pooled blood from these five mice (Figure 19).

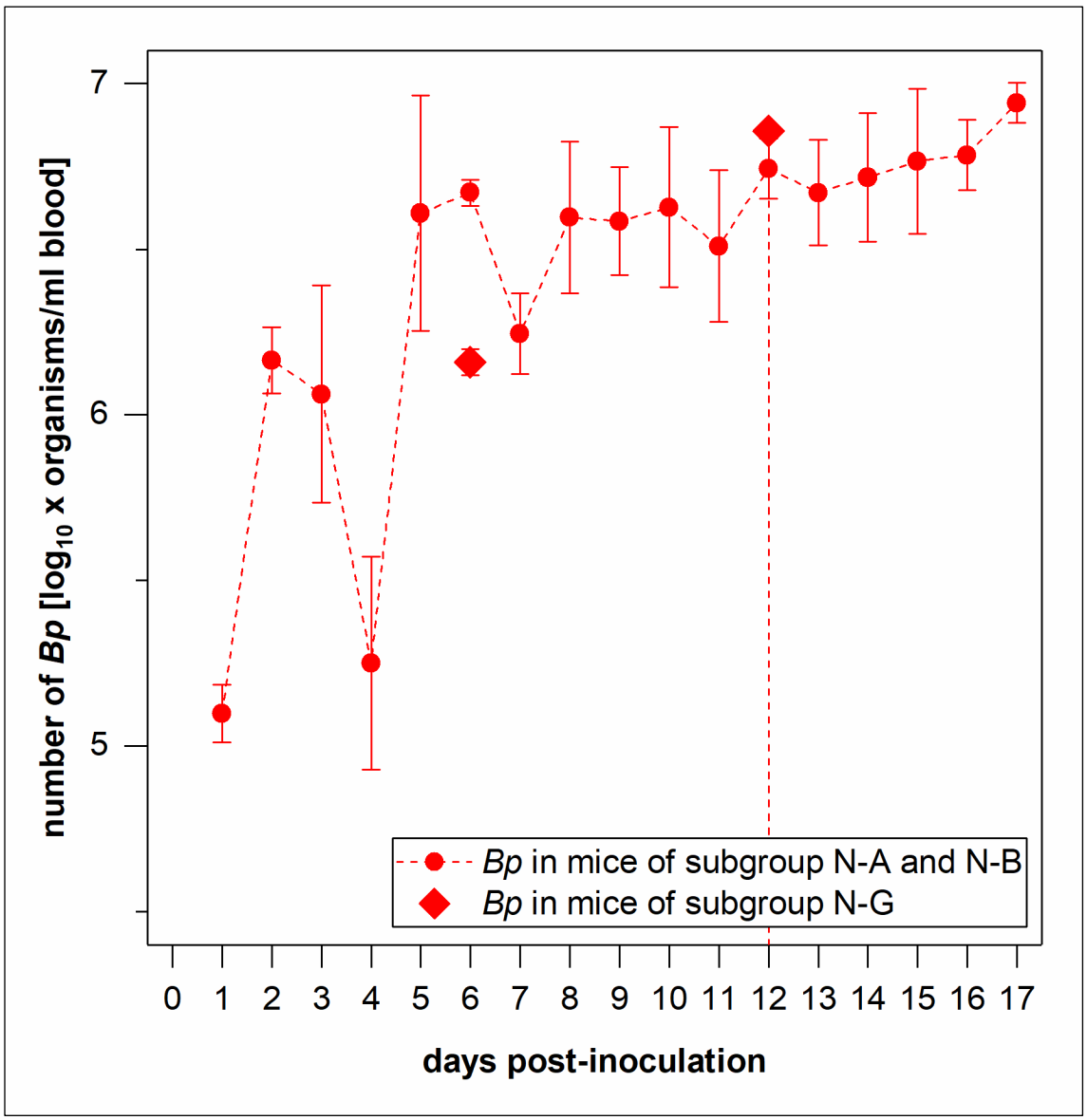

Figure 19: Kinetics of host-adapted *Bp* organisms in the blood of immunodeficient NOD-SCID mice

Kinetics of host-adapted *Bp* organisms in the blood of NOD-SCID mice (subgroup N-A and N-B) are shown with symbol ● during a 17-day infection period. Standard deviations between individual mice are indicated with the error bars. On day 12 p.i., 7.2 x 10^6 *Bp* spirochetes were detected per ml pooled blood (◆) from five mice in subgroup N-G.

1.2 Kinetics of host-adapted *Bb*ss spirochetes

After ID injection with cultured 1.0 x 10^6 (subgroup N-C and N-D) or 1.0 x 10^8 (subgroup N-E and N-F) *Bb*ss organisms, six NOD-SCID mice were monitored for motile spirochetes in the blood samples collected between day 3 and day 17. As shown in Figure 20, *Bb*ss organisms in the bloodstream was firstly detectable by qPCR (targeting *ospA* gene) on day 2 p.i. In the case of the inoculum dose of 1.0 x 10^6 *Bb*ss, the borrelial counts rose from 8.5 ± 1.4 x 10^3 cells/ml blood on day 2 up to 4.9 ± 3.3 x 10^4 cells/ml blood on day 7, followed by a small decrease on day 8 (2.6 ± 1.2 x 10^4 spirochetes/ml). On day 10, a second peak of spirochemia occurred with 3.9 ± 1.3 x 10^4 viable organisms per ml blood. Subsequently, borrelial concentration plateaued at ~ 3.0 x 10^4 cells/ml until day 17 (Figure 20). After ID injection of the higher inoculum dose of 10^8 *Bb*ss spirochetes, the first bacteraemia was noted on day 5 (7.2 ± 0.8 x 10^4 organisms/ml blood). On day 7 and day 11, 6.9 ± 2.1 x 10^4 and 4.9 ± 1.0 x 10^4 organisms circulated in each millilitre blood, respectively. At the other days, however, lower spirochete density was detected, which plateaued at ~ 4.0 x 10^4 (ranging from 2.3 x 10^4 to 3.9 x 10^4) per ml blood (Figure 20). According to statistical analyse, the highest spirochete densities in the blood caused by 10^6 and 10^8 *Bb*ss inoculum showed no significant difference ($p = 0.1958$). Considering the difficulty of centrifugation for higher *Bb*ss counts and the viability of spirochetes in the suspended medium, the inoculum dose of 10^6 was used for further experiments. Day 10 (3.9 x 10^4 cells/ml blood) was considered as the expected day with host-adapted and relatively peaking number of borreliae. As shown in Figure 20, 3.5 x 10^4 (subgroup N-H) and 2.8 x 10^4 (subgroup N-I) *Bb*ss organisms per ml were recorded in pooled blood of each five NOD-SCID mice on day 10 post injection.

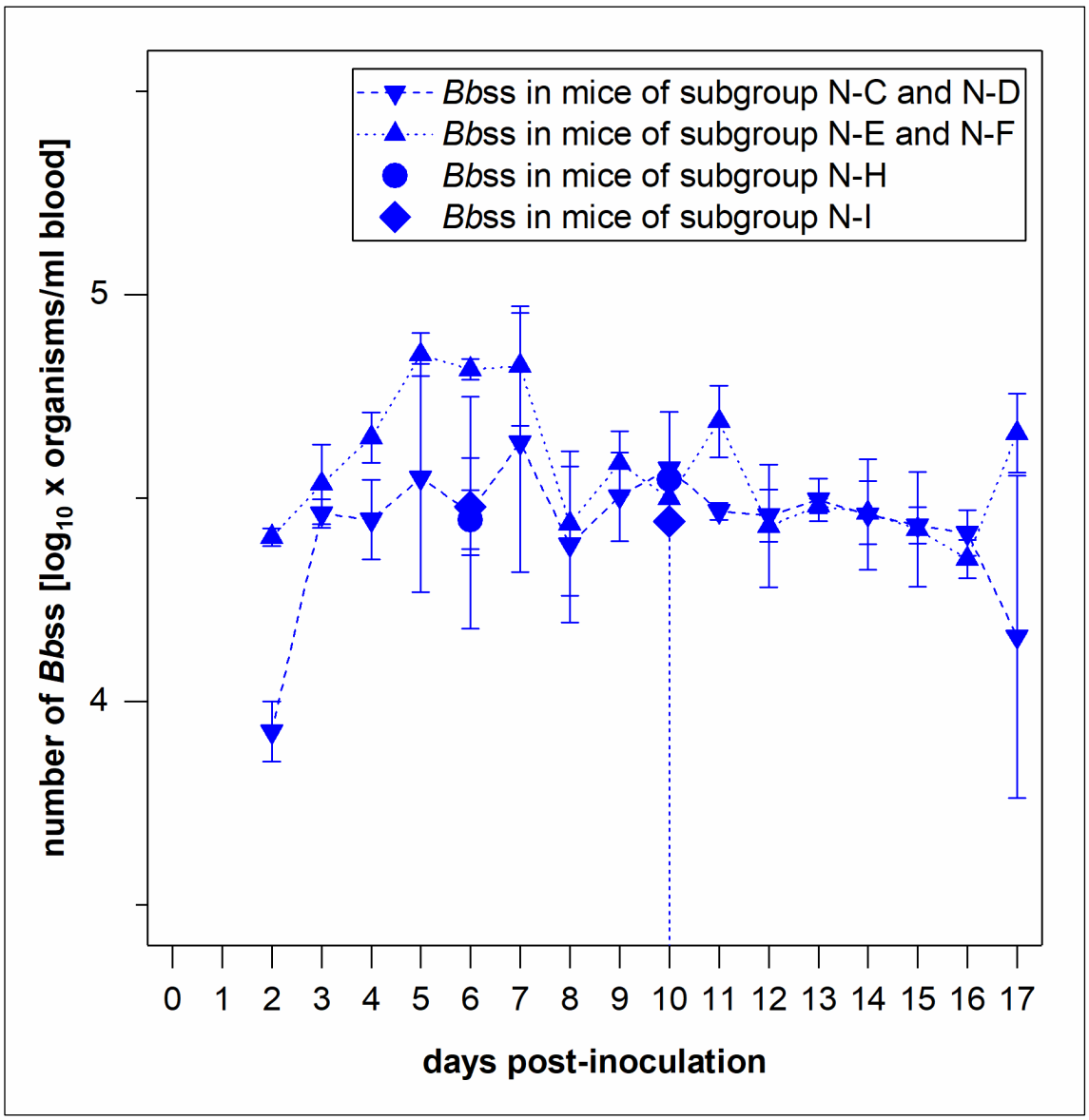

Figure 20: Kinetics of host-adapted *Bb*ss organisms in the blood of NOD-SCID mice

During a 17-day infection period, kinetics of host-adapted *Bb*ss organisms in the blood of NOD-SCID mice are shown with symbol ▼ (subgroup N-C and N-D with 1.0 x 10^6 *Bb*ss inoculum dose) and ▲ (subgroup N-E and N-F with 1.0 x 10^8 *Bb*ss inoculum dose). The standard deviations between individual mice are shown with the error bars. On day 10 p.i., 3.5 x 10^4 (●) and 2.8 x 10^4 (◆) *Bb*ss organisms per ml pooled blood from five mice in subgroup N-H and N-I were detected, respectively.

2 Distribution of borreliae in tissues of NOD-SCID mice

On day 12 and day 10 after injection of culture-derived 1.0 x 10^5 *Bp* and 1.0 x 10^6 *Bb*ss, respectively, in most of the collected tissue samples from NOD-SCID mice (subgroup N-G and N-H) the *flaB* (*Bp*) and the *ospA* (*Bb*ss) gene were detected by qPCR. Motile spirochetes were also identified by cultivation (Table 15). Briefly, *Bp* DNA was detected in all skin (infection areal) (5/5), tibiotarsal joint (5/5), spleen (5/5), urinary bladder (5/5), heart (5/5), and brain tissue (5/5) samples, but not in either of the five ear tissue samples (0/5). In subgroup N-H, each collected tissue sample (ear, skin, joint, spleen, bladder, heart, and brain) was positive for *Bb*ss DNA. Although contamination occurred to some tissue samples as shown in Table 15, motile spirochetes were demonstrated in cultures with following tissue samples: joint (1/5 with *Bp*; 4/5 with *Bb*ss), spleen (2/5 *Bp*; 3/5 *Bb*ss), bladder (3/5 *Bp*; 5/5 *Bb*ss), heart (2/5 *Bp*; 4/5 *Bb*ss), and brain (3/5 *Bp*; 5/5 *Bb*ss).

Table 15: Distribution of *Bp* and *Bb*ss in tissues of NOD-SCID mice

inoculum*		mouse no.	spirochetes in tissue samples detected by culture/qPCR (organisms/mg)						
			ear	skin	joint	spleen	bladder	heart	brain
Bp 1.0 x 10^5 ID	subgroup N-G n=5	N1	# / 0	# / 17	# / 199	+ / 873	+ / 74518	+ / 14017	+ / 3702
		N2	# / 0	# / 47690	# / 117	# / 19	# / 1743	# / 46102	# / 10721
		N3	# / 0	# / 11529	+ / 262	- / 3381	+ / 83700	+ / 23582	+ / 11929
		N4	# / 0	# / 1171	# / 574	+ / 1050	+ / 26254	# / 28797	+ / 2639
		N5	# / 0	# / 311	# / 24	# / 144	# / 131872	# / 6168	# / 1896
positive rate	**3 / 5**		**0 / 0**	**0 / 5**	**1 / 5**	**2 / 5**	**3 / 5**	**2 / 5**	**3 / 5**
***Bb*ss** 1.0 x 10^6 ID	subgroup N-H n=5	N6	# / 48	# / 41467	+ / 20395	+ / 22	+ / 266594	+ / 360	+ / 11
		N7	# / 522	# / 18795	+ / 37393	+ / 537	+ / 217600	# / 333	+ / 12
		N8	# / 3082	# / 16655	# / 2113	# / 14	+ / 2110835	+ / 357	+ / 15
		N9	# / 10372	# / 18435	+ / 40571	# / 3	+ / 1141583	+ / 1122	+ / 47
		N10	# / 4987	# / 15288	+ / 17693	+ / 992	+ / 384204	+ / 2057	+ / 12
positive rate	**5 / 5**		**0 / 5**	**0 / 5**	**4 / 5**	**3 / 5**	**5 / 5**	**4 / 5**	**5 / 5**

*, culture-derived borreliae
#, contaminated culture
+, positive with motile spirochetes in culture
-, negative with spirochetes in culture

3 Population kinetics of borreliae in the blood of immunocompetent C3H/HeOuJ mice

One hundred microliter pooled blood that contained host-adapted *Bp* or *Bb*ss organisms were injected ID or strictly IV into C3H/HeOuJ mice. Thereafter, blood samples were collected at defined time points throughout a 49/50-day study period. Spirochete numbers in the bloodstream were detected by qPCR testing.

3.1 Population kinetics of *Bp* spirochetes in blood

As described above, 7.2 x 10^5 host-adapted *Bp* spirochetes in 100 µl pooled blood were inoculated into mice of subgroup C-A (ID injection) and C-B (IV injection). Blood samples from all ID and IV challenged animals were screened for the *flaB* gene applying the qPCR method until day 15 p.i. (Figure 21). Correspondingly, viable spirochetes were cultured and observed applying dark-field microscope. In ID inoculated mice, spirochete concentration was 8.3 ± 1.9 x 10^5 and 9.4 ± 15.8 x 10^5 *Bp* cells/ml blood on day 6 and day 9, respectively. A second peak of spirochetemia occurred on day 12 with 1.9 ± 1.7 x 10^6 organisms/ml. On day 18, however, only one single mouse (no. C4) was detected to be positive for *Bp* with 7.4 x 10^4 organisms/ml blood. Similarly, the IV injected mice in subgroup C-B demonstrated two peaks of *Bp* populations: 3.7 ± 2.4 x 10^6 organisms per ml blood on day 6 and 4.1 ± 6.8 x 10^6 organisms per ml blood on day 12. Interestingly, one single mouse (no. C10) contained 2.8 x 10^5 and 3.8 x 10^5 *Bp* cells/ml blood on day 21 day 24, respectively (Figure 21). Between day 27 and day 49/50, however, all of the ID and IV inoculated mice were negative for borrelial DNA and no motile spirochetes were re-cultured in medium.

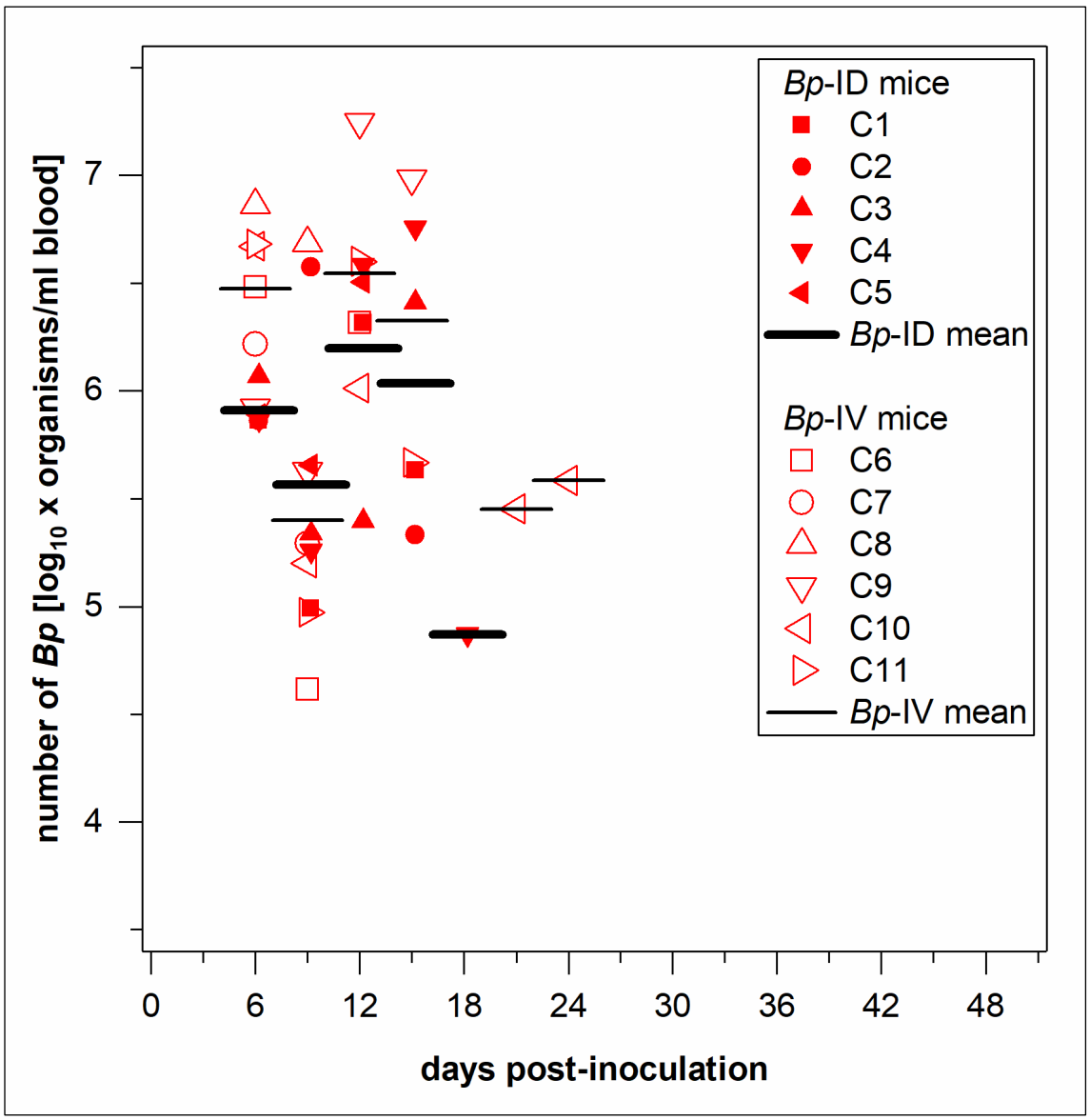

Figure 21: Kinetics of *Bp* spirochetemia in the blood of immunocompetent C3H/HeOuJ mice

3.2 Population kinetics of *Bb*ss spirochetes in blood

One hundred microliter pooled blood from NOD-SCID mice in subgroup N-H and N-I contained 3.5 x 10^3 and 2.8 x 10^3 *Bb*ss organisms, respectively. All blood samples from ID (subgroup C-C and C-E) or IV (subgroup C-D and C-F) injected mice produced no positive results for *Bb*ss-specific DNA. Spirochetes were not visible when dark-field microscope was applied. Cultivation of 30-µl blood from single mice or pooled blood from either ID or IV subgroup showed no growth of *Bb*ss spirochetes in 5 ml BSK-H medium.

4 Distribution of borreliae in tissues of C3H/HeOuJ mice

Tissue samples including ear, skin (inoculation areal), tibiotarsal joint, spleen, urinary bladder, heart, and brain were aseptically obtained from C3H/HeOuJ mice and separately cultivated in 10 ml of Pettenkofer/LMU *Bp* medium (for *Bp* at 37°C) or BSK-H medium (for *Bb*ss at 33°C) without any antibiotics. By qPCR testing and 6 weeks' cultivation, results were obtained.

4.1 *Bp* in tissues

Tissue samples were collected at the end of the observation period 49/50 days after spirochete inoculation. *Bp* organisms were detected in all brain tissue samples (100%) removed from C3H/HeOuJ mice that had received the bacteria either ID or IV. The other tissue samples (ear, skin, tibiotarsal joint, spleen, urinary bladder, and heart), however, produced neither viable spirochetes in the culture medium nor *flaB* gene signals in the qPCR tests. Bacterial contamination and overgrowth occurred in cultures with ear (10/11) and skin tissue samples (8/11; Table 16).

Table 16: Distribution of *Bp* in tissues of C3H/HeOuJ mice

inoculum*		mouse no.	spirochetes in tissue samples by culture/qPCR (organisms/mg)						
			ear	skin	joint	spleen	bladder	heart	brain
Bp 7.2 x 10^5 ID	subgroup C-A n=5	C1	# / 0	# / 0	- / 0	- / 0	- / 0	- / 0	+ / 39
		C2	# / 0	# / 0	- / 0	- / 0	- / 0	- / 0	+ / 31
		C3	# / 0	- / 0	- / 0	- / 0	- / 0	- / 0	+ / 89
		C4	# / 0	# / 0	- / 0	- / 0	- / 0	- / 0	+ / 59
		C5	# / 0	- / 0	- / 0	- / 0	- / 0	- / 0	+ / 136
positive rate	**5 / 5**		**0 / 0**	**0 / 0**	**0 / 0**	**0 / 0**	**0 / 0**	**0 / 0**	**5 / 5**
Bp 7.2 x 10^5 IV	subgroup C-B n=6	C6	# / 0	# / 0	- / 0	- / 0	- / 0	- / 0	+ / 30
		C7	# / 0	# / 0	- / 0	- / 0	- / 0	- / 0	+ / 34
		C8	- / 0	- / 0	- / 0	- / 0	- / 0	- / 0	+ / 75
		C9	# / 0	# / 0	- / 0	- / 0	- / 0	- / 0	+ / 216
		C10	# / 0	# / 0	- / 0	- / 0	- / 0	- / 0	+ / 63
		C11	# / 0	# / 0	- / 0	- / 0	- / 0	- / 0	+ / 74
positive rate	**6 / 6**		**0 / 0**	**0 / 0**	**0 / 0**	**0 / 0**	**0 / 0**	**0 / 0**	**6 / 6**

*, host-adapted borreliae
#, contaminated culture
+, positive with motile spirochetes in culture
-, negative with spirochetes in culture

4.2 *Bb*ss in tissues

On day 49/50 after ID inoculation of host-adapted spirochetes, 100% of the exposed mice (5/5, subgroup C-C; 6/6, subgroup C-E) were positive for *Bb*ss as shown by cultivation and qPCR testing of tissue samples (Table 17). Motile spirochete organisms were visualized in cultures containing tissue specimens from ears (4/11), skin samples (10/11), joints (11/11), spleen samples (4/11), bladders (11/11), hearts (11/11), and brains (2/11), except for the contaminated and negative cultures. Interestingly, *Bb*ss-specific DNA was detected only in urinary bladders (11/11) and heart tissue samples (7/11) of these *Bb*ss-ID inoculated animals (Table 17). Again, all 11 *Bb*ss-IV inoculated mice (subgroup C-D and C-F) tested completely negative for borrelial DNA and for motile *Bb*ss organisms in each tissue sample (Table 17).

Table 17: Distribution of *Bb*ss in tissues of C3H/HeOuJ mice

inoculum*		mouse no.	spirochetes in tissue samples by culture/qPCR (organisms/mg)						
			ear	skin	joint	spleen	bladder	heart	brain
***Bb*ss** 3.5 x 10^3 ID	subgroup C-C n=5	C21	# / 0	+ / 0	+ / 0	# / 0	+ / 875	+ / 0	- / 0
		C22	# / 0	+ / 0	+ / 0	+ / 0	+ / 676	+ / 0	+ / 0
		C23	+ / 0	+ / 0	+ / 0	+ / 0	+ / 1041	+ / 409	- / 0
		C24	# / 0	+ / 0	+ / 0	+ / 0	+ / 1867	+ / 0	+ / 0
		C25	# / 0	# / 0	+ / 0	+ / 0	+ / 1482	+ / 0	- / 0
positive rate	**5 / 5**		**1 / 0**	**4 / 0**	**5 / 0**	**4 / 0**	**5 / 5**	**5 / 1**	**2 / 0**
***Bb*ss** 3.5 x 10^3 IV	subgroup C-D n=5	C26	# / 0	- / 0	- / 0	- / 0	- / 0	- / 0	- / 0
		C27	# / 0	- / 0	- / 0	- / 0	- / 0	- / 0	- / 0
		C28	# / 0	# / 0	- / 0	- / 0	- / 0	- / 0	- / 0
		C29	# / 0	- / 0	- / 0	- / 0	- / 0	- / 0	- / 0
		C30	# / 0	- / 0	- / 0	- / 0	- / 0	- / 0	- / 0
positive rate	**0 / 0**		**0 / 0**	**0 / 0**	**0 / 0**	**0 / 0**	**0 / 0**	**0 / 0**	**0 / 0**
***Bb*ss** 2.8 x 10^3 ID	subgroup C-E n=6	C31	+ / 0	+ / 0	+ / 0	- / 0	+/ 2420	+ / 893	- / 0
		C32	+ / 0	+ / 0	+ / 0	- / 0	+/ 10295	+ / 501	- / 0
		C33	+ / 0	+ / 0	+ / 0	- / 0	+/ 1383	+ / 788	- / 0
		C34	# / 0	+ / 0	+ / 0	- / 0	+/ 2741	+ / 817	- / 0
		C35	# / 0	+ / 0	+ / 0	- / 0	+/ 906	+ / 109	- / 0
		C36	# / 0	+ / 0	+ / 0	- / 0	+/ 3487	+ / 607	- / 0
positive rate	**6/6**		**3 / 0**	**6 / 0**	**6 / 0**	**0 / 0**	**6 / 6**	**6 / 6**	**0 / 0**
***Bb*ss** 2.8 x 10^3 IV	subgroup C-F n=6	C37	- / 0	# / 0	- / 0	- / 0	- / 0	- / 0	- / 0
		C38	- / 0	# / 0	- / 0	- / 0	- / 0	- / 0	- / 0
		C39	- / 0	# / 0	- / 0	- / 0	- / 0	- / 0	- / 0
		C40	# / 0	# / 0	- / 0	- / 0	- / 0	- / 0	- / 0
		C41	# / 0	# / 0	- / 0	- / 0	- / 0	- / 0	- / 0
		C42	# / 0	# / 0	- / 0	- / 0	- / 0	- / 0	- / 0
positive rate	**0 / 0**		**0 / 0**	**0 / 0**	**0 / 0**	**0 / 0**	**0 / 0**	**0 / 0**	**0 / 0**

*, host-adapted borreliae
#, contaminated culture
+, positive with motile spirochetes in culture
-, negative with spirochetes in culture

5 Specific antibodies against *Bp* and *Bb*ss in C3H/HeOuJ mice

Plasma and serum samples were obtained from the blood samples of C3H/HeOuJ mice post ID or IV inoculation. Serological testing (ELISA or/and LIA) was applied to characterize the kinetics of antibody levels and the quality of the specific antibody response induced by borrelial spirochetes.

5.1 Kinetics of specific antibody response against *Bp*

As shown in Figure 22, ID and IV inoculation of host-adapted *Bp* organisms into C3H/HeOuJ mice elicited positive antibody responses. Clearly, *Bp*-ID mice generated similar antibody levels to that of the *Bp*-IV counterparts. In subgroup C-A and C-B, antibody levels rose to 267.5 and 166.1 KELA units on day 21 and decreased to 214.1 and 104.9 KELA units on day 24, respectively. Afterwards, antibody responses increased steadily and reached 300.1 (ID) and 264.5 (IV) KELA units on day 49/50 (end of this study). Serum samples obtained on the final day of the experiment were tested individually for each mouse. Calculated mean values were 296.6 ± 103.1 KELA units for *Bp*-ID and 208.7 ± 42.5 KELA units for *Bp*-IV.

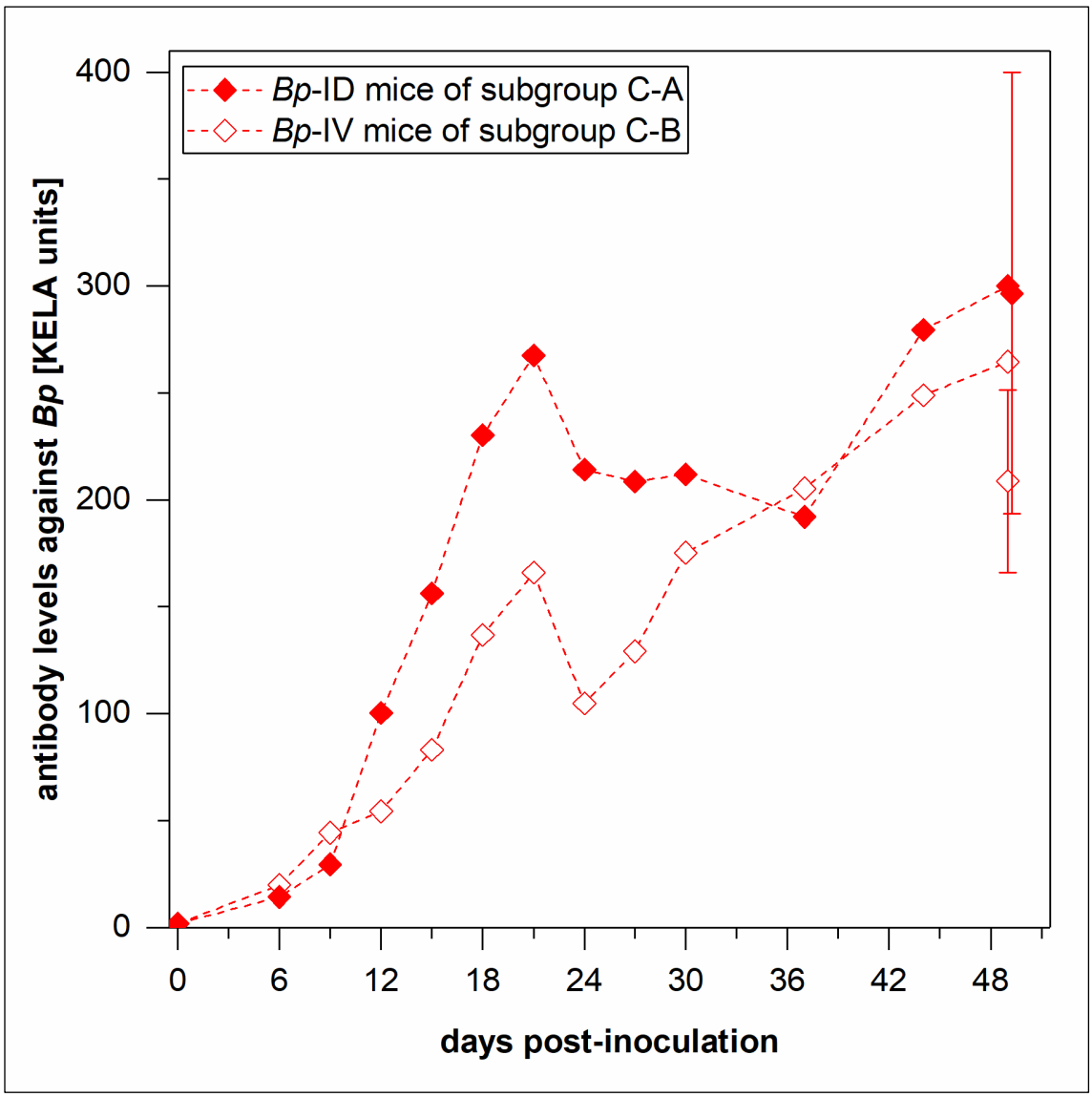

Figure 22: Kinetics of specific antibody response against *Bp* in C3H/HeOuJ mice

5.2 Kinetics of specific antibody response against *Bb*ss

Mice that had been injected intradermally with host-adapted *Bb*ss organisms (*Bb*ss-ID) developed specific antibodies with a steady increase (Figure 23). In subgroup C-C and C-E, the antibody levels increased similarly up to 493.7 and 526.9 KELA units, respectively, on day 49/50 p.i. In contrast, none of IV-injected mice (subgroup C-D and C-F) produced specific antibodies against *Bb*ss. Only approx. 30 KELA units were recorded, ranging from 13.7 to 59,8. Serum samples from individual mice clearly showed specific IgG and IgM antibody responses: 478.9 ± 54.9 KELA units (*Bb*ss-ID-1, subgroup C-C), and 492.7 ± 68.8

KELA units (*Bb*ss-ID-2, subgroup C-E), 31.3 ± 25.5 KELA units (*Bb*ss-IV-1, subgroup C-D) and 28.6 ± 17.1 KELA units (*Bb*ss-IV-2, subgroup C-F).

No data are available from mouse no. C23 in subgroup C-C, because the final blood collection via intracardiac puncture failed.

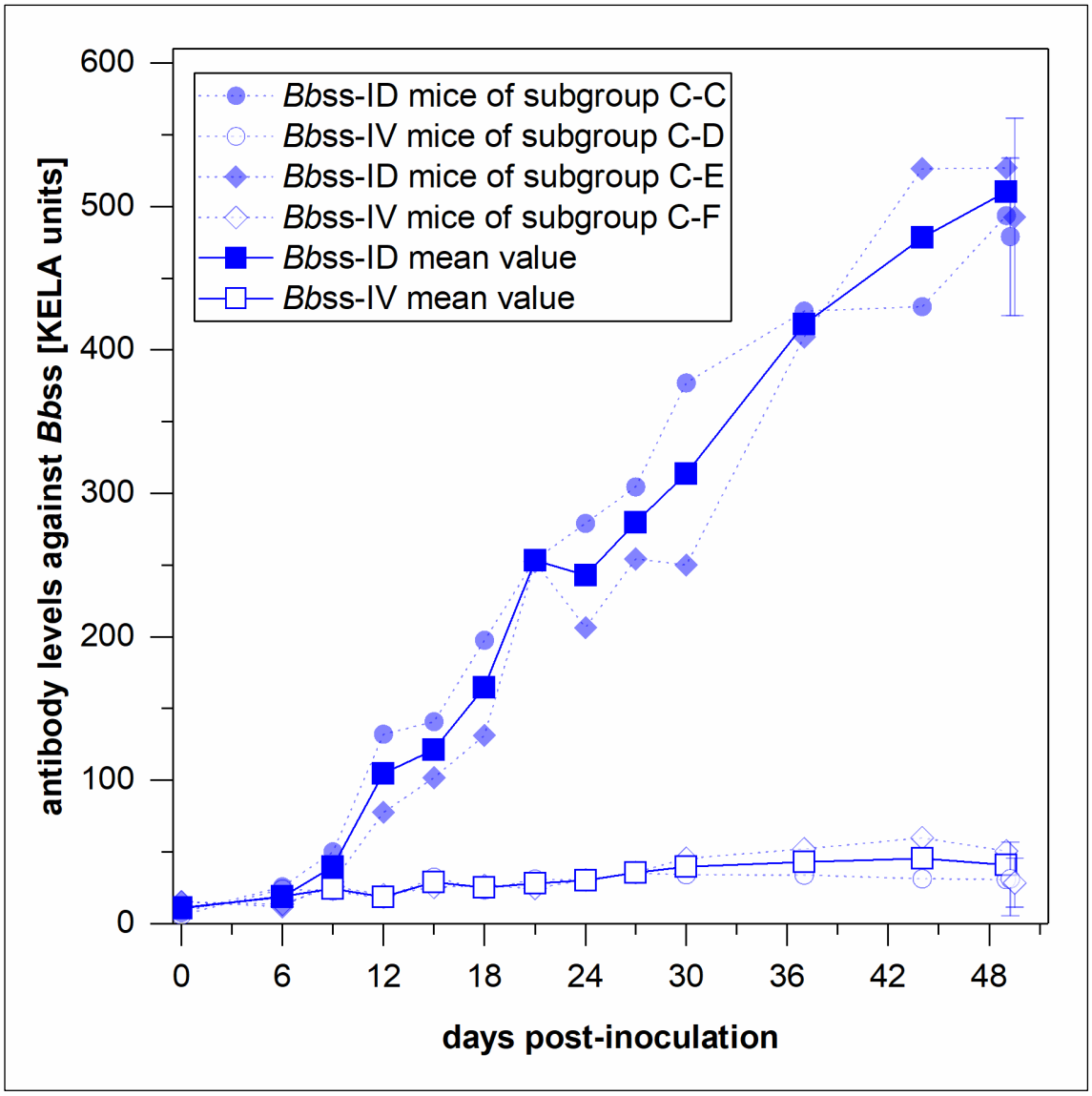

Figure 23: Kinetics of specific antibody response against *Bb*ss in C3H/HeOuJ mice

5.3 Visualization of specific antibodies against *Bb*ss

As shown in Figure 24, individual serum samples obtained from 11 mice inoculated intravenously with *Bb*ss (5 in subgroup C-D and 6 in subgroup C-F) produced no specific signals on the line immunoblot (lanes 4 to 14). In contrast, serum samples collected from ten animals inoculated intradermally with *Bb*ss (4 in subgroup C-C, because no serum was from mouse no. C23; and 6 in subgroup C-E) reacted with following antigens (lanes 15 to 24): strong signals with VlsE mix (10/10); strong/moderate signals with OspC mix (8/10), BmpA (7/10) and p58 (58 kDa; 5/10); weak signals with DbpA mix (10/10) and p83 (83 kDa; 8/10).

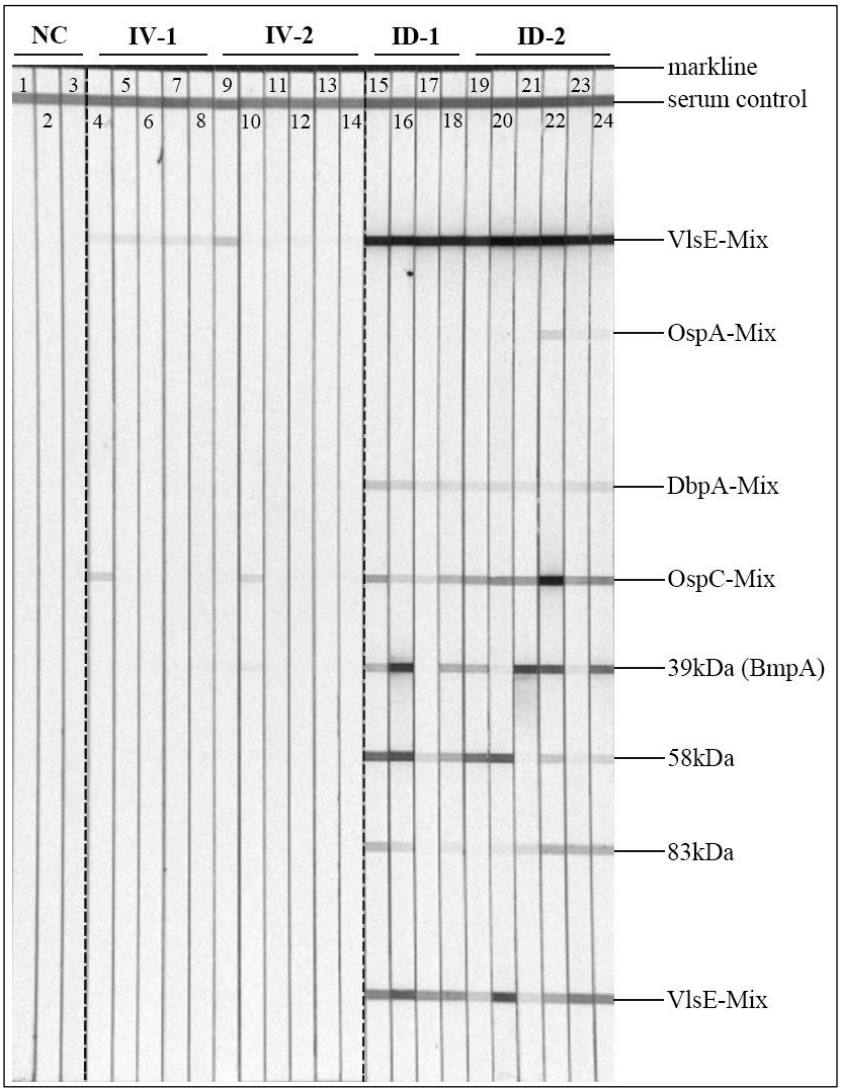

Figure 24: Representative IgG line immunoblots of individual serum samples from *Bb*ss inoculated C3H/HeOuJ mice

Lanes belong to the individual serum samples in the following groups: NG (lanes 1-3) to plasma samples from three of five mice in subgroup C-NG; IV-1 (subgroup C-D) to lanes 4-8; IV-2 (subgroup C-F) to lanes 9-14; ID-1 (subgroup C-C, except for one mouse no. C23) to lane 15-18; ID-2 (subgroup C-E) to lanes 19-24.

VI DISCUSSION

Tick-borne relapsing fever (TBRF), although recognized for ages, remains one of the neglected diseases with only a few studies clarifying the interactions between host, tick and pathogens (TALAGRAND-REBOUL et al., 2018). Among its pathogens, *Bp* can cause acute infection and make TBRF a widespread disease in the large areas of Middle East and Central Asia. This bacterium poses a noteworthy health threat not only to the local residents and the military but also to the tourists who may carry the infection back to non-endemic countries on their return journey (SIDI et al., 2005; DE VERDIERE et al., 2011; KUTSUNA et al., 2013). However, to the best of our knowledge, data on the pathogenesis of *Bp* in the vertebrate host are still limited, though previous studies have developed animal models with *Bp* infection via ID or IP inoculation (ASSOUS et al., 2006; SCHWARZER et al., 2016). On the other hand, Lyme borreliosis (LB) is the most common tick-borne disease in the northern hemisphere, but continuously needs to be extensively investigated because of its long-lasting infections in humans (STONE & BRISSETTE, 2017; SPRONG et al., 2018). *Bb*ss, the endemic species causing LB in the USA and Europe, has been the focus of scientific research in the last years. However, some authors still hold different views on its dissemination route in the vertebrate host. In the context of the characterization of the exact dissemination pathway of both TBRF and LB borreliae in mammals, there is to date no suitable animal model available in which specific organisms are injected into the venous system for long-term infection. With the intention of further insights into pathogenic strategies of *Borrelia*, research in the underlying dissemination is of great importance. Therefore, we established the present murine model using ID and strict IV inoculation of host-adapted *Bp* and *Bb*ss organisms into immunocompetent mice. Evident clarifications from our study are as follows. Firstly, *Bp* causes persistent infection in the immunocompetent mice regardless of the ID or IV inoculation route. This indicates its excellent adaptation to the circulation environment and its robust capability of surviving in the bloodstream. During the course of the infection, *Bp* spirochetes disseminate hematogenously to distant organs (especially brain), causing persistent infection. Secondly, *Bb*ss, in comparison to *Bp*, cannot lead to a systemic infection via IV inoculation, because these spirochetes in the bloodstream are probably unable to cross the blood vessel and thereafter translocate to distal tissues. However, LB spirochetes are able to migrate to distant tissues primarily via non-hematogenous pathways (i.e., tissue migration shown after ID

inoculation) and establish systemic infections in tissues such as ear, skin, joint, bladder and heart. The distribution of *Bb*ss organisms in our ID-inoculated mice also resembles the scenario that was observed in human tissue specimens with LB infection (DURAY & STEERE, 1988; DURAY, 1989). Finally, our murine model could be a valuable research tool in understanding the pathogenesis of TBRF and LB borreliae during human and animal infections. Nevertheless, our data provide insight into potential risk factors that come with blood transfusions in clinical practice. Transfusion of *Bp* contaminated blood is highly problematic, because of its potential to cause spirochetemia and persistent brain infection in recipients. On the contrary, the risk of receiving *Bb*ss infection via blood transfusion is low or should be considered to be non-existent.

1 Host-adapted *Bp* and *Bb*ss organisms

In our study, immunodeficient NOD-SCID mice were used to generate host-adapted borrelia organisms. After ID injection of 1.0×10^5 in vitro cultured *Bp* spirochetes, hematogenous organisms were first detected on day 1 (the first sampling time point p.i.) with $1.3 \pm 0.2 \times 10^5$ cells per ml blood. Along the infection time, *Bp* burden kept rising up to $8.8 \pm 1.2 \times 10^6$ cells/ml blood (on day 17-end time point), except for some slight decrease on day 4, 7 and 11 (see Figure 19). By contrast, *Bb*ss organisms were first detectable on day 2 with $8.5 \pm 1.4 \times 10^3$ and $2.5 \pm 0.1 \times 10^4$ cells per ml blood of NOD-SCID mouse after ID inoculation of 1.0×10^6 and 1.0×10^8 cultured spirochetes, respectively (see Figure 20). The highest density recorded was $7.2 \pm 0.8 \times 10^4$ *Bb*ss per ml blood, although a high inoculum dose (1.0×10^8) was used. Given that *Bp* organisms (1.0×10^5) were injected in a 10- and 10^3-fold lower dose than *Bb*ss, it seems that *Bp* adapts more rapidly to the circulation than *Bb*ss and proliferates efficiently in the bloodstream of immunodeficient animals.

On day 12 and day 10 post injection of cultured *Bp* (1.0×10^5 cells) and *Bb*ss (1.0×10^6 cells), respectively, most sampled tissue samples collected from the ears, skin (site of inoculation), tibiotarsal joints, spleens, urinary bladders, hearts, and brains from NOD-SCID mice presented with spirochetes by in vitro cultivation and qPCR (see Table 15). Therefore, spirochetes of both *Bp* and *Bb*ss can disseminate to various distant tissues of immunodeficient animals. For the first time, our study reported the population dynamics of *Bp* in the bloodstream and its distribution in different tissues of immunodeficient mice, which complete data from a prior study (SCHWARZER et al., 2016) and also results we obtained with immunocompetent mice (see Figure 21 and Table 16).

As described above, on day 12 and day 10, 7.2 x 10^6 *Bp* and 3.5 x 10^4 or 2.8 x 10^4 *Bb*ss organisms per ml blood were harvested from NOD-SCID mice, respectively. After ID inoculation of 100 µl pooled blood that contained these host-adapted *Bp* (7.2 x 10^5) or *Bb*ss (3.5 x 10^3 or 2.8 x 10^3) organisms, persistent infection was induced in all immunocompetent mice as confirmed by the detection of viable spirochetes in various tissue samples collected at the end of the observation periods (see Tables 16 and 17) and the induction of consistently rising *Borrelia*-specific antibody levels (see Figures 22 and 23). These evidences are substantial to prove that host-adapted borrelia organisms are invasive and pathogenic to the immunocompetent host even though a low inoculum dose of *Bb*ss was used. However, this relatively low number of borreliae may reflect more faithfully the actual pathophysiological conditions in terms of a low spirochete load (~ 10^3 to 10^5 or less) being transmitted from the tick to the host or from the infectious host to the naïve tick (BURKOT et al., 1993; GOLDE et al., 1994; DE SILVA & FIKRIG, 1995; KERN et al., 2011; GRILLON et al., 2017). It is also reasonable to explain why many studies dealing with TBRF and LB borreliae widely utilize an injection dose of approx. 10^3 - 10^6 spirochetes. According to clinical case studies, *Bb*ss was positively re-isolated from large quantity of plasma or blood sample volume (9 - 18 ml) from patients at early stage of LB infection (WORMSER et al., 1998; MARASPIN et al., 2001; WORMSER, 2006; LIVERIS et al., 2011). This indicates that the actual spirochete number in blood of the LB patients is extremely low, which was estimated to be 0.1 cultivable cell/ml in whole blood (WORMSER, 2006). Compared to LB infection, numerous *Bp* organisms were found in blood of naturally infected animals and humans (DE VERDIÈRE et al., 2011; RAFINEJAD et al., 2011; KUTSUNA et al., 2013; BANETH et al., 2016). Consequently, the injection doses used for host-adapted *Bp* and *Bb*ss spirochetes in this project can be considered as practical and optimal for further experiment as scheduled in this study. Furthermore, experimental results based on the host-adapted bacteria appear to be more reliable and reproducible compared to those using in vitro cultured bacteria as stated by Woodman et al. (WOODMAN et al., 2009).

2 Intravenous inoculation via jugular vein but not tail vein

According to the published data, the lateral tail vein of the rat or mouse was preferentially utilized for IV inoculation with the help of a suitable restrainer (Figure 25) (STEEL et al., 2008). However, this method is considered challenging and often unsuccessful (YARDENI et al., 2011). Firstly, it is technically difficult to visualize the vessel. The fragile tail vein

shows a poor visibleness through the dark brown skin of the C3H/HeOuJ mouse used in our study. Injection errors may occur. For example, the needle may accidentally punch through the vessel walls due to a small size of the tail vein. Secondly, a low number of bacteria adhering to the tip of the needle might be unavoidably carried off into the surrounding tissue or skin during the process of removing the apparatus from the mouse's tail. Unpublished data from an earlier experiment, which was independently carried out of the present study, showed an overall successful rate of 93.1% (67 of 72 C3H mice) on IV injection via tail vein.

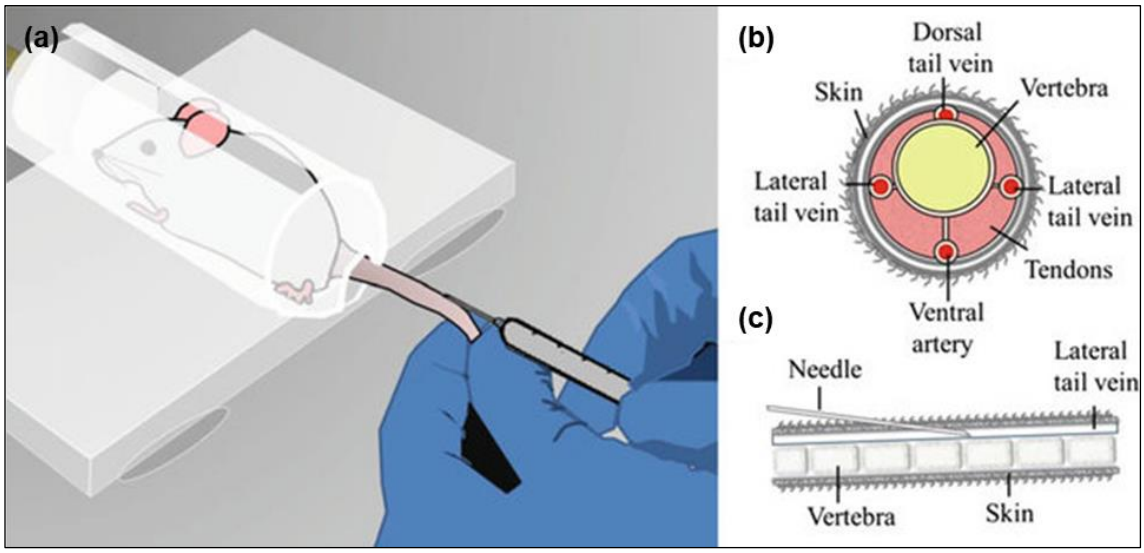

Figure 25: Demonstration for mouse lateral tail vein injection

(a) mouse restrainer and tail vein injection technique, **(b)** axial section of mouse tail anatomy, **(c)** sagittal cross section of mouse lateral tail with needle in position
Cited from Brown et al. (BROWN et al., 2018)

Therefore, an alternate route of IV injection was considered. The jugular vein, in comparison to other vessels such as femoral vein and retro-orbital plexus, is easier to be separated from the connective tissue and exposed clearly for further procedures carried out under the constant surveillance with a stereo microscope (Figure 16). Many investigators use catheters into jugular vein for intravenous administration or chronic/acute implantation (BARR et al., 1979), although it is necessary to ligate the vein to avoid bleeding while puncturing it with a needle (POPOVIC & POPOVIC, 1960).

In our experiment, the right jugular vein was ligated. This procedure does not affect the blood-brain-barrier or the blood circulation in mice as previously described (SAKATA et al., 1999; ATKINSON et al., 2012). Special sterile instruments/materials such as Vannas-style

spring scissors, blunt micro hook and Alzet mouse jugular catheter made great contributions to the strict IV inoculation without producing severe damage to the vessel wall. Flushing with 50 µl sterile isotonic saline avoided dead space in the catheter to ensure that the complete blood volume and spirochete dose was applied. Above all, the visual observation of the whole procedure during injecting, flushing, and removing was clearly visible using stereo microscopy with optimal magnification. The success rate of the injection into the jugular vein was 100% (6/6 for *Bp*, 11/11 for *Bb*ss) without any bleeding of blood into surrounding tissues. On day 49/50 after intravenous inoculation of host-adapted borreliae into the immunocompetent C3H/HeOuJ mice, *Bp* organisms induced noticeable antibody levels and brain infections were detected consistently (see Figure 22 and Table 16) whereas neither *Bb*ss-specific-antibody levels nor motile *Bb*ss spirochetes were detectable (see Figure 23 and Table 17). Thus, *Bb*ss organisms, although host-adapted and invasive as demonstrated by the ID inoculation, are unlikely to cross the blood vessels for further infection of distant sites in C3H/HeOuJ mice. The clear results of *Bp* and *Bb*ss infection via IV inoculation are strong evidence for our successful microvasculature surgery without any blood/borreliae leakage.

However, previous studies claim that hematogenous dissemination of *Bb*ss is still the spreading route used by this organism. The careful reading of the publication reveals that mice were exposed to *Bb*ss by an intraperitoneal injection due to unsuccessful IV technique (GABITZSCH et al., 2006). Obviously, the results obtained by an IP but not IV inoculation may lead to a misinterpretation of the data, but do not clarify how *Bb*ss disseminates from the injection site to the distant tissues. Other studies used huge doses (4×10^8 organisms per animal) and culture-derived *Bb*ss for IV inoculation (MORIARTY et al., 2008; NORMAN et al., 2008). Even though they indicated the hematogenous spread based on few interactions of spirochetes and the blood vessels under shear force condition, only a small area was visualized within a short time period (5 - 45 min). The puzzling results of these studies with the large inoculum dose were also argued by some authors (HYDE et al., 2011). It should be mentioned that under optimal conditions *Borrelia* spirochetes reach cell densities of $\sim 10^8$ to 10^9 per ml in culture medium (AGUERO-ROSENFELD et al., 2005; PAPPAS et al., 2011). However, only few spirochetes are naturally deposited in the dermis of the mammals after tick attachment as described above. Hence, both the strict IV inoculation and a reasonable inoculum dose play the most crucial roles to establish a reliable murine model and to draw a realistic conclusion.

3 Spirochetemia and brain infection of *Bp* in C3H/HeOuJ mice

A hematogenous dissemination of some TBRF *Borrelia* spp. (e.g., *B. hermsii*, *B. duttonii*, and *B. turicatae*) from the bloodstream to distant tissue sites in the mammals has been described (CADAVID & BARBOUR, 1998; DWORKIN et al., 2008). Previous studies on the infection dynamics have all described spirochetemia regardless of the TBRF spirochete or inoculation route: *Bp* (ID and IP), *B. hermsii* (ID, IP, IV, and SB[4]), *B. turicatae* (ID), and *B. miyamotoi* (blood transfusion) (HOVIS et al., 2006; BENOIT et al., 2010; RAFINEJAD et al., 2011; BOYLE et al., 2014; DICKINSON et al., 2014; KRAUSE et al., 2015; SCHWARZER et al., 2016). In our study, numerous *Bp* organisms in blood of C3H/HeOuJ mouse were detectable until day 24 after either ID or IV inoculation with 7.2×10^5 host-adapted organisms (see Figure 21). Fluctuations of spirochetemia during the infection period in our ID infected mice, which are consistent with previous data (SCHWARZER et al., 2016), were similarly observed in our IV challenged animals (see Figure 21). For example, higher-level spirochetemia was detected at day 6 and day 12 followed by a small decrease until day 9 and day 15, respectively. Our first bleeding time point at day 6 showed $8.3 \pm 1.9 \times 10^5$ *Bp* cells/ml blood after ID injection while $3.7 \pm 2.4 \times 10^6$ were seen after IV injection. Taking into account that $\sim 1.5 \times 10^5$ (at day 1 p.i.) and $\sim 4 \times 10^6$ (at day 6 p.i.) *Bp* organisms/ml blood was reported in the prior study (SCHWARZER et al., 2016), this suggests that *Bp's* entry from the inoculation sites into the circulation is a rapid event, followed by a rapid proliferation in the bloodstream of immunocompetent animals. This suggestion has been supported by other researchers, who found that transmission and dissemination of *B. turicatae*, another TBRF *Borrelia*, occurred during the short time required for tick engorgement (BOYLE et al., 2014).

Correlated with the fluctuations of spirochetemia, *Bp*-specific antibodies appeared in both ID and IV inoculated mice and the antibody level increased steadily until day 21 followed by a slight decline until day 24. Although no spirochetes were detected by qPCR or in vitro cultivation from day 24 until days 49/50 p.i., the antibody level in both ID and IV infected mice rose again during this period (see Figure 22). A reasonable explanation might be that the immune response is permanently triggered by antigen provided by a persistent infection. All cerebral tissue samples collected on day 49/50 p.i. were positive for *Bp* (see Table 16). However, antibodies even at a high level were not able to eliminate *Bp* organisms present in

[4] SB: subcutaneous inoculation

brain tissue – probably an immune-privileged site. *Bp* spirochetes seem to sustain a persistent infection in the brain while at the same time they induce no tissue damage or clinical signs (SCHWARZER et al., 2016).

4 *Bb*ss' lack of spirochetemia in C3H/HeOuJ mice

In our study, neither *Bb*ss DNA detection nor re-cultivation of vital spirochetes was possible in any blood specimen of C3H/HeOuJ mice regardless of ID or IV inoculation of host-adapted *Bb*ss organisms (3.5×10^3 or 2.8×10^3). Similarly, some murine studies have found that LB spirochetes can only occasionally or even not at all be reisolated from peripheral blood of experimental immunocompetent animals, though a persisten infection was established by high (10^8; SB), or low (10^3, 10^4; ID) inoculum doses of *Bb*sl organisms (SIMON et al., 1991; HODZIC et al., 2003; LIU et al., 2004). Although previous data reported low yield of *Bb*sl borreliae by culturing blood/plasma from some LB patients at the early infection stage (WORMSER et al., 1998; MARASPIN et al., 2001; WORMSER, 2006; LIVERIS et al., 2011), no scientific data so far have shown whether these low number of blood-borne spirochetes travel to distal tissues and cause persistent infection there. According to our hypothesis, strict IV inoculated *Bb*ss spirochetes mimic closely the LB organisms in infected mammalian hosts, including humans. However, neither *Bb*ss spirochetes in blood or tissues (see Table 17) nor *Bb*ss-specific antibodies (see Figure 23 and 24) were detected in IV challenged C3H/HeOuJ mice. Consequently, *Bb*ss organisms cannot survive in the bloodstream or disseminate further to other tissues. Possibly the spirochetes are cleared by the complement system, which plays a critical role in controlling early infection before the specific humoral immune response is induced (STEERE et al., 2016). This can also explain why no specific antibodies against these host-adapted *Bb*ss cells were detected during the entire infection period of our study. It should be questioned, whether the low density of blood-carrying LB borreliae in human patients should be considered the same as the high-level spirochetemia of TBRF organisms, which can disseminate from the bloodstream to distant sites in the body (e.g., brain). On the contrary, our intradermally injected animals produced *Bb*ss-induced specific antibodies during the infection period (see Figure 23 and 24). On day 49/50 (the end of the observation period), motile spirochetes were also isolated from most tissue samples such as ear, skin (inoculation site), urinary bladder and tibiotarsal joint (see Table 17). Interestingly, some tissue samples showed positive spirochetes in the culture but no *Bb*ss DNA was detectable in the same

tissue. Reasons might be: 1) no *Bb*ss DNA was extracted from the tissue (weight ≤ 50 mg); 2) *Bb*ss-specific DNA presented in the extracted DNA sample was present at a very low concentration that was below the detection limit of the applied qPCR. This phenomena could be explained by the finding that even one single viable spirochete may be recovered by culture but it is not possible to detect DNA from one single cell by PCR (BARBOUR, 1984). Unlike *Bp*, our infectious *Bb*ss spirochetes appear to only infrequently target the brain tissue but prefer to persist in tissues such as skin, bladder, joint, and heart of the immunocompetent mice, consistent with prior publications dealing with LB infection in humans and animals (SHIH et al., 1992; DIVAN et al., 2018).

Infection with LB spirochetes initially begins after the deposition of the bacteria into the dermis by the vector tick (*Ixodes* spp.). Presumably, infection may remain locally at this site until it is cleared by antibiotic treatment or by strong immunological responses in the mammalian hosts. Alternatively, the borrelial organisms may disseminate to other cutaneous sites and/or more significantly to extracutaneous sites, such as the joints, nervous system, or heart (STEERE et al., 1987; GRILLON et al., 2017). During this dissemination, only few spirochetes may accidentally disseminate into the bloodstream. However, as indicated by our results based on IV inoculation, these blood-borne borreliae are not capable of leaving the bloodstream for further tissue infection. On the contrary, the spirochetes injected in the dermis caused persistent infection in various tissues. Therefore, the blood circulation is not significantly involved during the dissemination. Instead, skin and connective soft tissues probably serve as critical intermediate media for spirochete spread and spirochete tissue migration. This view is further supported by the fact that immunosuppression with dermocorticoid clobetasol reactivated borrelia in the skin tissue while the blood still remained spirochete-negative (GRILLON et al., 2017). In contrast, high-level spirochetemia was achieved during TBRF infection and brain tissue colonization by inducing immunosuppression (LARSSON et al., 2006). Moreover, early studies have determined some *Bb*sl proteins, e.g., DbpA, DbpB, and BBK32, which may contribute to spirochete migration and colonization through mediating tissue adherence (GUO et al., 1995; GUO et al., 1998; PROBERT & JOHNSON, 1998). As clarified by other studies, extracellular matrix and connective tissues, which are especially rich in collagen, provide a protective niche for migration and persistence of Lyme disease spirochetes (CABELLO et al., 2007).

In summary, infectious *Bb*ss spirochetes, which induced persistent infection via ID but not via IV inoculation, probably disseminates via a non-hematogenous route, i.e., via tissue

migration. In fact, certain observational and experimental data from a couple of other studies support the idea of tissue migration used by *Bb*ss organism (HANSEN & LEBECH, 1992; BERGLUND et al., 1995; STRAUBINGER et al., 1997; MOTAMENI et al., 2005; CABELLO et al., 2007; TUNEV et al., 2011).

5 Diagnosis and treatment of TBRF and LB infection

As indicated from our results and other studies as mentioned above, blood samples from TBRF borrelia infected patients can be used for diagnostic test, especially those characterized with high fever attacks and a history of tick exposure (DWORKIN et al., 2008). The dark field or fluorescence microscope can be useful tools to observe spirochetes in the blood. Molecular methods, especially PCR, identifies most *Borrelia* spp. by amplification and analysis of species-specific markers in their genomic DNA (FUKUNAGA et al., 1996; SAFDIE et al., 2010). However, spirochetes with a low density in the blood, especially during afebrile periods of TBRF infection in patients, can be missed by microscope and limited to PCR tests. Therefore, serologic confirmation of TBRF infections is further performed, most frequently by ELISA and western blot tests as used in our and prior studies (DWORKIN et al., 2008; SCHWARZER et al., 2016). However, some limitations still occur to the serodiagnosis of TBRF infection in patients and naturally infected animals. It is known that the serologic assays are based on the antigen of whole-cell lysate of the in vitro cultured spirochetes. Therefore, false-positive reactions may be possible in the ELISA and IFA, partially caused by reactive epitopes on the spirochete's flagellin protein, which presents in other borrelial species as well (MAGNARELLI et al., 1984; MAGNARELLI et al., 1987a). Additionally, serologic test can be challenging due to antigenic variations of TBRF spirochetes during infection in various mammals, including humans (DWORKIN et al., 2008). In addition, residual brain infection as shown by our and other results (SCHWARZER et al., 2016) ask for new diagnostic approaches, especially when no biopsy specimens are available. Once infected with *Bp*, early treatment strategies can be carried out with intravenous medication of antibiotics, if oral administration is not tolerated (DWORKIN et al., 2008). Importantly, antimicrobial medicines that can penetrate the blood-brain-barrier are necessary for the treatment of brain infection.

In terms of *Bb*ss infection, our murine model shows clearly the fact that diagnostic procedures based on routinely blood detection, either culture or molecular assay or both, produce most likely unreliable or negative results. The skin rash EM, the most common and

earliest clinical manifestation of LB infection in humans, is considered as the only evidence that enables a reliable clinical diagnosis of this disease (STRLE & STANEK, 2009). Spirochetes can also be culture-confirmed from this skin lesion, which expands as LB borreliae migrate away from the site of the tick bite (NADELMAN et al., 1996). Without treatment or in the course of an insufficient treatment LB spirochetes are able to establish chronic (month- to year-long) infections in skin tissue of mice and dogs (BARTHOLD et al., 1993; KRUPKA & STRAUBINGER, 2010). In autopsy/biopsy specimens and human skin lesions even months to years after initial LB infection, small numbers of *Bb*sl borreliae have been detected. The remaining spirochetes in the skin suggest their direct role in the generation and perpetuation of clinical signs of LB (BARTHOLD et al., 1991). Investigations on *B. afzelii* in wild and laboratory rodents have revealed that the spirochete load in the skin regulates the success of host-to-tick transmission (RÅBERG, 2012; REGO et al., 2014; JACQUET et al., 2015). Studies from Grillon et al. also suggested that naïve nymphs acquire the low density of spirochetes exclusively from the local skin and not from the blood (GRILLON et al., 2017). Therefore, skin biopsy specimens close to the tick bite or even distal site (e.g., ear tissue), are most suspicious for detection of LB borrelia infection during early or even late stages. As shown in our study, *Bb*ss was re-isolated from skin (injection areal) and even ear tissue (distal for skin) after needle inoculation. However, not all patients present the skin lesion EM and some patients infected or co-infected with other pathogens [e.g., Southern tick-associated rash illness (STARI); Masters' disease] also show similarity to EM (MASTERS et al., 1998; MASTERS et al., 2008). Some patients are even asymptomatic during *Bb*sl infection. Consequently, diagnosis avoiding false-negative or false-positive should be carried out further based on detection of corresponding antibodies. Our serological results (see Figure 23 and 24) indicate obviously that a two-tiered test including ELISA and LIA is reliable for confirmation of a successful *Bb*ss infection in the mammals (CDC, 1995; WILSKE et al., 2000).

According to some investigations (SHIH et al., 1992; KNAUER et al., 2011), the topical treatment with penicillin G, amoxicillin, ceftriaxone, doxycycline, and azithromycin-containing formulation effectively cleared *Bb*sl spirochetes and stopped the infection when these antibiotics were applied on murine skin shortly after tick exposure or needle inoculation. Therefore, topical treatment strategies with proper antibiotics on deposition site are of particular importance to discontinue the spread of LB spirochetes (KNAUER et al., 2011). In the context of persistent infection of LB borreliae in host tissues, however, a major

question, whether antibiotics used are able to eliminate the spirochetes hiding in these immune-privileged sites, is still in need to answer.

6 Prospect

The in vivo model described in this dissertation provides profound insights into the hematogenous and tissue-bound pathways of dissemination followed by *Bp* and *Bb*ss organisms, respectively, which play significant roles in pathogenetic mechanism in mammalian hosts.

The successful initiation of persistent infections by 7.2 x 10^5 *Bp* organisms after IV inoculation into C3H/HeOuJ mice reveals clearly the risk of transfusion-transmitted infections (TTI) humans face when they receive such treatment. Injection of only 100 *Bp* organisms can induce high-level spirochetemia (~ 10^6 organisms/ml blood) and persistent brain infection (SCHWARZER et al., 2016). Based on the ability to survive and proliferate in the blood circulation, it is reasonable to presume that even such a low inoculum dose can induce infection in immunocompetent individuals when transfused in case of an emergency. Hence, physicians should be aware of the potential danger in blood donation and transfusion. On the contrary, no TTI of LB borreliae has been reported so far. And according to our data, the medical risk of receiving this infection by blood transfusion can be considered as non-existent. Distinct clinical signs of LB, i.e. LA, ACA, and LNB, are thought to be associated with distinct genospecies of *Bb*ss, *B. afzelii*, and *B. garinii*, respectively. Therefore, further attempts are necessary to explore and compare the dissemination capacities of these species for better understanding how LB spirochetes interact with mammalian hosts and cause diseases.

Furthermore, the recent discovery of new borrelia species such as *B. miyamotoi*, a relapsing fever spirochete that is transmitted by hard-shell ticks (e.g., *I. ricinus*) needs to be considered as an additional risk factor for humans and animals. Further studies are needed to describe in detail the exact route this organism may take to invade distant sites in mammalian hosts.

VII SUMMARY

Borrelia persica (*Bp*) is the most prevalent species causing tick-borne relapsing fever (TBRF) in Central Asia and the Middle Eastern countries whilst *B. burgdorferi* sensu stricto (*Bb*ss) is an endemic genospecies causing Lyme borreliosis (LB) in North America and Europe. TBRF patients commonly suffer from recurrent fever attacks due to spirochete proliferation and antibody-mediated killing of these organisms in the blood, whereas during the early stage of infection LB patients commonly show skin lesions (erythema migrans). If left untreated, LB patients may develop to a chronic phase with Lyme arthritis or other tissue lesion due to inflammatory responses (e.g., acrodermatitis chronica atrophicans). Despite a severe debate over the exact dissemination pathways employed by borrelia organisms, little is known how *Bp* and *Bb*ss disseminate in the body of mammalian hosts. Hence, a murine model with a novel and precise infection approach was established to explore the dissemination route of host-adapted *Bp* and *Bb*ss organisms post intradermal (ID) and intravenous (IV) inoculation in immunocompetent C3H/HeOuJ mice.

Since borreliae are able to express differentially outer surface proteins (Osps) in vitro and in vivo, it was necessary to generate the host-adapted bacteria from immunodeficient NOD-SCID mice. An ID inoculation of 1.0×10^5 culture-derived *Bp* organisms per animal resulted in the peak of spirochetemia with 8.8×10^6 cells per ml blood of NOD-SCID mouse. However, 1.0×10^6 and 1.0×10^8 cultured *Bb*ss caused spirochetemia with a low load resulting in up to 4.9×10^4 and 7.2×10^4 organisms per ml blood, respectively. Interestingly, hematogenous *Bp* spirochetes were detectable by both qPCR and cultivation at least one day earlier when compared to *Bb*ss. It seems that *Bp* is more efficient in entering the bloodstream and multiplying in the circulation of the immunodeficient mice than *Bb*ss.

Compared to the ID injection the strict IV inoculation via the jugular vein in C3H/HeOuJ mice was the most critical step to clarify whether host-adapted *Bp* and *Bb*ss organisms are capable of leaving the bloodstream for further colonization in mammalian tissues. After either ID or IV inoculation of 7.2×10^5 host-adapted *Bp* organisms, these spirochetes were detectable up to day 24 in C3H/HeOuJ mice. *Bp* counts reached up to 1.9×10^6 and 4.1×10^6 per ml blood sampled at day 12 post ID and IV challenge, respectively. Correspondingly, antibody detection with collected plasma and serum samples showed that specific antibodies to *Bp* were induced. Notably, all brain tissue samples from both ID and IV inoculated

C3H/HeOuJ mice were positive for *Bp* when tested with molecular methods and cultivation. In contrast, neither the *Bb*ss *ospA* gene nor viable spirochetes were found in the blood of any C3H/HeOuJ mouse inoculated either ID or IV with approx. 3 x 10^3 host-adapted *Bb*ss spirochetes. Interestingly, on day 49/50 after ID injection, *Bb*ss spirochetes were re-isolated from most tissue samples collected from the ears, skins (injection areal), tibiotarsal joints, urinary bladders, and hearts. Strong antibody responses against *Bb*ss were detected in all ID inoculated animals. In contrast, after IV injection of host-derived *Bb*ss organisms, neither borrelial DNA nor viable spirochetes were present in any tissue sample collected from these animals. In addition, they did not produce specific antibodies against *Bb*ss.

In summary, 1) *Bp* is excellently capable of adapting to and surviving in the bloodstream. The spirochete disseminates predominantly via hematogenous route to distant organs (e.g., brain) to establish a persistent infection; 2) *Bb*ss is a tissue-bound spirochete that migrates primarily via non-hematogenous routes. Intradermal deposition will cause a successful persistent infection while the blood vessel system is an impasse for this bacterium. The animal model applied in this study mimics the natural infection conditions of tick bite as closely as possible. Borreliae were used in an inoculum dose expected to be deposited by ticks in the skin of mammal hosts or in a development phase (host-adapted) they most likely express in blood. The detailed characterization of the spirochetes' dissemination pathways provides an advanced understanding of the pathogenicity mechanism of TBRF and LB species, and can help to improve diagnostic approaches or therapeutic strategies. Notably, blood transfusion of *Bp* may poses a high risk of infection to recipients whereas the risk to become infected with *Bb*ss is nonexistent.

VIII ZUSAMMENFASSUNG

Borrelia persica (*Bp*) zählt zu den verbreitetsten Spirochätenarten, die Rückfallfieber (tick-borne relapsing fever, TBRF) in Zentralasien und Ländern des Mittleren Ostens verursacht, während *Borrelia burgdorferi* sensu stricto (*Bb*ss) als endemische Genospezies in Nordamerika und Europa vorkommt und die Lyme-Borreliose (LB) verursacht. TBRF-Patienten leiden häufig unter wiederkehrenden Fiberattacken, ausgelöst durch Spirochätämie und die dadurch verursachte Antikörper-mediierte Bekämpfung der Erreger. LB-Patienten hingehen zeigen in der frühen Krankheitsphase in der Regel nur eine charakteristische Hautläsion (Erythema migrans). Unterbleibt die Behandlung der LB-Patienten, kann sich die Infektion auf Grund der anhaltenden Entzündungsprozesse zur chronischen LB (z. B. acrodermatitis chronica atrophicans) weiterentwickeln. Obwohl eine angeregte Diskussion zur Verbreitung der Spirochäten, ob über den Blutkreislauf oder über das Gewebe, zu den verschiedenen Borrelien vorherrscht, ist wenig über deren genauen Verbreitungsmechanismen bekannt. Deshalb wurde in dieser Studie ein Mausmodell mit einem neuartigen und präzisen Injektionsmethode etabliert, um den Verbreitungsweg von zuvor wirtsadaptierten *Bp*- oder *Bb*ss-Organismen nach intradermaler (ID) und strikter intravenöser (IV) Inokulation in immunkompetenten C3H/HeOuJ-Mäusen zu untersuchen.

Da Borrelien in der Lage sind, ihre Oberflächenproteine (Osps) unter in-vitro- und in-vivo-Bedingungen unterschiedlich zu exprimieren, war es zunächst notwendig, wirtsadaptierte Borrelien aus ursprünglich kulturadaptierten Bakterien mithilfe von immundefizienten NOD-SCID-Mäusen zu erzeugen. Dafür wurden die Tiere einerseits intradermal *Bp*-Organismen ($1{,}0 \times 10^5$) inokuliert, was zu einer Spirochätämie mit bis zu $8{,}8 \times 10^6$ Zellen/ml Blut in den Mäusen führte. Die intradermale Injektion von $1{,}0 \times 10^6$ bzw. $1{,}0 \times 10^8$ aus der Kultur gewonnenen *Bb*ss-Organismen resultierte hingegen in einer Spirochätämie auf niedrigem Niveau ($4{,}9 \times 10^4$ und $7{,}2 \times 10^4$ Zellen/ml). Zudem waren *Bp*-Spirochäten im Blut mit Hilfe der qPCR und Kultivierung mindestens einen Tag früher nachweisbar als *Bb*ss. Insoweit scheint es, dass *Bp* im Vergleich zu *Bb*ss besser dazu in der Lage ist, in den Blutkreislauf von immundefizienten Mäusen einzudringen und sich dort zu vermehren.

Im Vergleich zur ID-Injektion war die strenge IV-Inokulation über die Jugularvene der C3H/HeOuJ-Maus der kritischste Schritt, um zu klären, ob wirtsadaptierte *Bp*- und *Bb*ss-Organismen in der Lage sind, den Blutkreislauf für eine Besiedlung der Gewebe im Säugetierwirt zu verlassen. Nach ID- oder IV-Inokulation von $7{,}2 \times 10^5$ Wirts-adaptierten

Bp-Organismen waren die Spirochäten im Blut der C3H/HeOuJ-Mäusen bis zum Tag 24 nachweisbar. Die Zahl der *Bp* erreichte Höchstwerte von bis zu 1,8 x 10^6 und 4,1 x 10^6 pro ml Blut am Tag 12 nach der ID- und IV-Inokulation. Dementsprechend wurden spezifische Antikörper gegen *Bp* in Plasma- und Serumproben der Mäuse nachgewiesen. Mit Hilfe molekularer Methoden und Kultivierung konnte gezeigt werden, dass insbesondere alle Hirngewebeproben der C3H/HeOuJ-Mäusen sowohl nach ID- als auch nach IV-Injektion positiv für *Bp* waren. Im Gegensatz dazu wurden weder das *Bb*ss-*ospA*-Gen noch lebende Spirochäten im Blut aller C3H/HeOuJ-Mäuse gefunden, die ca. 3 x 10^3 wirtsangepasste *Bb*ss-Spirochäten erhalten hatten. Allerdings wurden am Tag 49/50 nach der ID-Injektion *Bb*ss-Spirochäten aus den meisten Proben, die aus Ohr-, Haut (Injektionsfläche), Tibiotarsalgelenk-, Harnblase- und Herzgewebe entnommen wurden, isoliert. Eine robuste Antikörperreaktion gegen *Bb*ss wurden in allen ID-inokulierten Mäusen nachgewiesen. Im Gegensatz dazu konnten in Gewebeproben der IV-inokulierten Tieren weder Borrelien-DNA noch lebensfähige Spirochäten detektiert werden. Eine spezifische Antikörperreaktion gegen *Bb*ss blieb in diesen Tieren völlig aus.

Zusammenfassend wurde in dieser Studie gezeigt, dass 1) *Bp* sich hervorragend an den Blutstrom der Wirte anpassen und darin überleben kann. Danach verbreitet sich das Bakterium vorwiegend auf hämatogenem Weg zu entfernt gelegenen Organen (z. B. Gehirn), um eine persistierende Infektion zu etablieren; 2) *Bb*ss ist ein gewebegebundenes Bakterium, das sich hauptsächlich durch Gewebe fortbewegt und sich so im Körper des Wirtes verbreitet. Das intradermale Einbringen der *Bb*ss-Organismen führt zu einer erfolgreich etablierten persistierenden Infektion, während die intravenöse Injektion eine Sackgasse für dieses Bakterium darstellt. Hervorzuheben ist insbesondere, dass das hier vorgestellte Mausmodell die natürlichen Infektionsbedingungen nach Zeckenbiss so gut wie möglich durch die Verwendung einer Injektionsdosis nachahmt, die nach Zeckenstich in der Haut eines Wirtes zu erwarten ist. Zudem wurden wirtsadaptierte Spirochäten verwendet, die wie in dieser Studie vorliegenden Entwicklungsphase so auch im Blut der Wirte vorkommen sollten.

Die detaillierte Beschreibung der Ausbreitungswege ermöglicht ein besseres Verständnis der Pathogenitätsmechanismen sowohl der TBRF- als auch der LB-Spirochäten und kann dazu beitragen, die Diagnose- und Behandlungsstrategien für die durch diese Spirochäten ausgelösten Krankheiten verbessern. Insbesondere ist anzumerken, dass das Risiko sich nach Bluttransfusion mit *Bp* sich zu infizieren hoch ist, während das Risiko sich mit *Bb*ss zu infizieren vernachlässigbar ist.

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XI APPENDIX

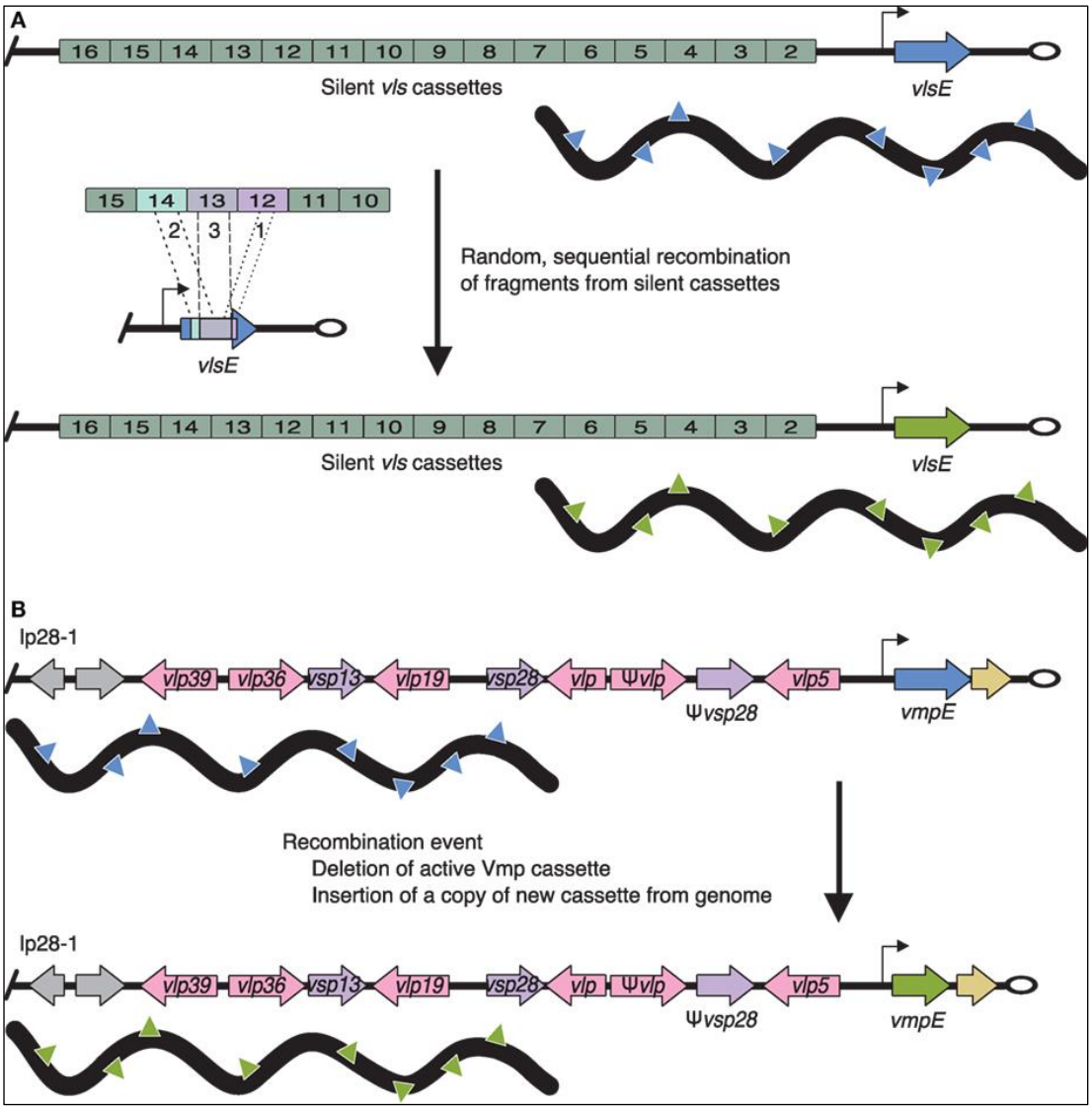

Figure A1: Antigenic variation mechanisms in Lyme and Relapsing fever *Borrelia*

(A) VlsE: the expression locus (*vlsE*) is located near the telomere (open oval) of linear plasmid (lp) 28-1 (blue or green arrow, promoter is indicated by a black arrow). Silent vls cassettes are located upstream and in the opposite orientation of *vlsE*. Antigenic variation occurs through the random and sequential insertion of silent cassette fragments (labeled 1, 2, and 3).
(B) *vlp* (pink arrows) and *vsp* (purple arrows) cassettes are located throughout the genome on lp28-1, 28-2, 28-3, 28-4, and 32-1. The expression locus (blue or green arrow, promoter is indicated by a black arrow) is found on lp28-1 near the telomere (open oval). Changing the expressed Vmp cassette is achieved through deletion of the current cassette (blue arrow) followed by insertion of a copy of a new cassette (green arrow *via* recombination events) resulting in a change in the expressed Vmp on the surface of the bacterium (denoted by blue or green triangles, respectively). Gray arrows indicate non-Vmp ORFs; tan arrows indicate downstream homology sequences (DHS, sequences found throughout the genome and required for mapping recombination events at the Vmp expression locus).
Data from Stone and Brissette (STONE & BRISSETTE, 2017)

Table A1: Clinical characteristics, common manifestations, and laboratory support in the diagnosis of LB

Clinical characteristics (by state of infection)	**common manifestations**	**essential laboratory evidence**	**supporting laboratory evidence**
erythema migrans (early localized[a] and disseminated[b] infection)	expanding red or bluish-red patch (≥ 5 cm in diameter) with or without central clearing; advancing edge is typically distinct, often intensely coloured, and not noticeably raised	none if typical	culture from skin biopsy; seroconversion of specific serum IgG antibodies or presence of specific IgM[c]
borrelial lymphocytoma (a rare manifestation, localized infection)	painless bluish-red nodule or plaque, usually on ear lobe, ear helix, nipple, or scrotum; more frequent in children (especially on ear) than in adults	specific IgG antibodies	histology; culture from skin biopsy
Lyme neuroborreliosis (early disseminated infection)	meningitis, cranial neuritis, radiculitis (motor or sensory), meningoradiculitis	lymphocytic pleocytosis in CSF; intrathecally produced specific antibodies[d]	intrathecal total IgM and/or IgG synthesis; specific oligoclonal bands in CSF; seroconversion of specific serum IgG antibodies[c]; culture from CSF
Lyme neuroborreliosis (persistent[e] infection)	chronic encephalomyelitis, demyelinating-like syndrome, axonal polyneuropathy, cognitive and behavioral changes	lymphocytic pleocytosis in CSF; intrathecally produced specific antibodies[d]; specific serum IgG antibodies	specific oligoclonal bands in CSF
cardiac Lyme disease (a rare manifestation, early disseminated infection)	acute onset of atrioventricular (I–III) conduction disturbances, rhythm disturbances, and sometimes myocarditis or pericarditis; alternative explanations should be excluded	significant change in levels of specific IgG antibodies[c]	culture from endomyocardial biopsy

Lyme arthritis (early disseminated and persistent infection)	recurrent attacks or persisting objective joint swelling in one or more large joints; alternative explanations should be excluded	high level of specific serum IgG antibodies	detection of borrelial DNA in synovial fluid and/or tissue (culture from synovial fluid and/or tissue)
acrodermatitis chronica atrophicans (persistent infection)	long-standing red or bluish-red lesions, usually on the extensor surfaces of extremities; initial doughy swelling; lesions eventually become atrophic; possible skin induration and fibroid nodules over bony prominences	high level of specific serum IgG antibodies	histology; culture from skin biopsy

[a] Duration of infection is days to ~ 4 weeks.
[b] Duration of infection is weeks to months (range, 1 - 14 months).
[c] Specific antibody levels in serum may increase in response to progression of infection or treatment, or may decrease due to abrogation of the infection process. Samples collected a minimum of 3 months apart may be required in order to detect a decrease in IgG levels.
[d] Intrathecally produced specific antibodies are determined by investigating simultaneously drawn samples of CSF and serum.
[e] Duration of infection is months to years.
Data modified from references (PAROLA & RAOULT, 2001; STANEK et al., 2012; STANEK & STRLE, 2018)

Table A2: Treatment regimens for tick-borne relapsing fever

medication	drug[a]	dose for adults	interval (times daily)	treatment duration
oral	Chloramphenicol	500 mg	4	7 - 10 days
	Doxycycline	100 mg	2	
	Erythromycin	500 mg	4	
	Tetracycline	500 mg	4	
parenteral[b]	Chloramphenicol	500 mg	4	
	Doxycycline	100 mg	2	
	Erythromycin	500 mg	4	
	Penicillin G (procaine)	600,000 IU	1	
	Tetracycline	250 mg	4	

[a] Most commonly used antibiotics are in bold.
[b] Parenteral medication should be continued until oral medication is tolerated. If oral medication is tolerated at the time of diagnosis, parenteral medication may not be necessary.
Data modified from references (DWORKIN et al., 2008; KUTSUNA et al., 2013)

Table A3: Antibiotic treatment regimens available for Lyme borreliosis

route	drug[a]	dose for adults	dose for children	treatment duration[b]
oral therapy	**Doxycycline** (patients ≥ 8yrs)	100 mg 2× day	4 mg/kg (up to 100 mg) 2× day	14 - 28 days
	Amoxicillin	500 mg 3× day	50 mg/kg (up to 500 mg) 3× day	
	Cefuroxime axetil	500 mg 2× day	30 mg/kg (up to 500 mg) 2× day	
intravenous therapy	**Ceftriaxone**	2 g 1× day	50 - 75 mg/kg (up to 2 g) 1× day	
	Cefotaxime	2 g every 8 hrs	150 - 200 mg/kg (up to 2 g) every 8 hrs	
	Penicillin G	18 - 24 million U/day divided every 4 hrs	200,000 - 400,000 U/kg daily divided every 4 hrs (up to 18 - 24 million U/day)	

[a] Most commonly used antibiotics are in bold.
[b] Duration of treatment is based mainly on the clinical manifestations of LB.
Data modified from references (MURRAY & SHAPIRO, 2010; STANEK et al., 2012)