A STING from a Tick:

Epidemiology, Ecology and Clinical Aspects of Lyme Borreliosis

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Abstract

Lyme borreliosis (LB) is the most common tick-borne disease in the Northern Hemisphere and the number of LB cases is increasing. The infection is caused by spirochetes belonging to the *Borrelia burgdorferi* sensu lato complex, and is, in Europe, transmitted to humans by *Ixodes ricinus* ticks.

To gain a deeper knowledge of the interactions between ticks, humans and *Borrelia* bacteria, we investigated temporal differences in exposure to tick bites in different parts of Sweden and the Åland Islands, Finland during the years 2008 and 2009. We also investigated the site of tick attachment on the human body and the time it takes for a person to detected and remove such ticks. Furthermore, the distribution of *Borrelia* species and the number of *Borrelia* cells in the ticks were investigated. Sera taken from the tick-bitten persons at study inclusion were analyzed for the presence of *Borrelia* antibodies. Three months later, the clinical outcome and the serological response of the tick-bitten persons were investigated. A total of 2154 *I. ricinus* ticks and 1546 participants were included in the studies.

Participants were exposed to tick bites between April and November, but temporal and spatial differences in exposure to ticks was found. The majority of the tick bites were caused by nymphs (70%) and most tick bites took place on the legs (50%). The site of tick attachment on the body as well as the age and gender of the participant influenced how soon a tick was detected. The majority of participants removed "their" ticks later than 24 hours of attachment. Of all ticks, 26% was *Borrelia*-infected, but the prevalence varied between the life stages of the tick and between the studied areas. Six species of the *B. burgdorferi* sensu lato complex and one *Borrelia* species that may cause tick-borne relapsing fever were detected. Adult ticks that had

fed more than 36 hours contained a lower number of *Borrelia* cells than adult ticks that had fed less than 36 hours. The seroprevalence among the participants varied between genders as well as between the studied areas. Of all participants, 2% was diagnosed with LB and 2.5% seroconverted without an LB diagnose. A correlation between seroconversion and duration time of tick attachment was found, but the number of *Borrelia* cells in the tick, did not explain the risk of infection for the bitten person.

A deeper knowledge and a better understanding of the interactions between ticks, humans and *Borrelia* bacteria may contribute reducing the risk for tick bites and the risk of developing LB after a tick bite.

Swedish summary

Lyme borrelios (LB) är den vanligaste fästingburna infektionen på norra halvklotet. Infektionen orsakas av bakterier som tillhör gruppen *Borrelia burgdorferi* sensu lato-komplexet och överförs till människor via fästingbett. Det dröjer i allmänhet minst ett dygn från det att fästingen har bitit sig fast, till dess att borreliabakterier överförs. Fästingarten *Ixodes ricinus*, som har tre aktiva livsstadier (larv, nymf och adult fästing), är i Sverige välkänd som överförare av de sjukdomsframkallande borreliabakterierna. Andelen borreliainfekterade fästingar i Sverige som biter människa är dock okänt och man vet heller inte vilka borreliaarter dessa fästingar bär på eller om antalet borreliabakterier i sådana fästingar förändras med fästingens blodsugningstid. LB är inte anmälningspliktig i Sverige och man vet inte hur många personer som årligen får en borreliadiagnos. Tidigare studier pekar på att det kan röra sig om mellan 5 000 och 10 000 fall per år. Denna uppskattning är baserad på 20 år gammal data och sen dess har fästingpopulationen i Sverige ökat. Med den växande fästingpopulationen har även risken för fästingbett hos människor och risken att drabbas av LB ökat.

Syftet med avhandlingen var att få en djupare förståelse för samspelet mellan fästingar, människor och borreliabakterier. Under 2008 och 2009 undersökte vi när på året personer från olika delar av Sverige och på de Åländska öarna blev fästingbitna. Vi undersökte även vilka livsstadier av fästingen de blev bitna av. Vidare undersökte vi om dessa fästingar var borreliainfekterade och vilka borreliaarter de bar på. Antalet borreliabakterier i fästingarna kvantifierades och korrelerades till hur länge de sugit blod. Vi undersökte även om de fästingbitna personerna hade IgG-antikroppar i blodet strax efter bettillfället. Tre månader efter fästingbettet undersökte vi det kliniska och

serologiska utfallet hos deltagarna genom att analysera nya blodprov, enkäter och deltagarnas patientjournaler. Totalt deltog 1546 fästingbitna personer från tre olika områden i Sverige samt de Åländska öarna och 2154 *I. ricinus* fästingar som sugit blod från människa analyserades för förekomst av borreliabakterier.

Deltagarna blev bitna av fästingar mellan april och november men både säsongsmässiga och geografiska skillnader i exponering av fästingbett upptäcktes. Majoriteten av fästingbetten orsakades av nymfer (72 %) och de flesta betten inträffade på benen (50 %). Majoriteten av deltagarna (63 %) upptäckte och avlägsnade fästingarna mer än 24 timmar efter att fästingen börjat suga blod. Fästingens bettställe på kroppen, samt ålder och kön hos den fästingbitne personen, påverkade tiden för att upptäcka och avlägsna fästingen. Fästingar som bitit sig fast i huvudet och i underlivet avlägsnades i regel senare jämfört med fästingar som bitit sig fast på andra delar av kroppen. Äldre personer och män avlägsnade i regel fästingarna senare jämfört med yngre personer och kvinnor som blivit fästingbitna. Var fjärde fästing (26 %) var borreliainfekterad men detta varierade både mellan fästingens olika livsstadier (0-36%) och mellan de undersökta områdena (11-31%). Totalt detekterades sex arter av *B. burgdorferi* sensu lato-komplexet samt en borreliaart som kan orsaka fästingburen återfallsfeber. Vi detekterade ett lägre antal borreliabakterier hos adulta fästingar som sugit blod från människa i mer än 36 timmar jämfört med adulta fästingar som sugit blod kortare tid. Av samtliga deltagare diagnostiserades 2 % för LB och 2.5 % hade en pågående borreliainfektion (serokonversion) men blev inte diagnostiserade för LB. Vi fann att personer som serokonverterade tog bort sina fästingar betydligt senare (58 h) än de som inte serokonverterade (29 h). Däremot fann vi inget samband mellan antalet borreliabakterier i fästingen och det serologiska svaret hos de fästingbitna. IgG seroprevalensen hos de fästingbitna deltagarna varierade mellan könen (16

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% av kvinnorna och 27 % av männen) och även mellan de undersökta områdena (17-23%).

Resultaten från detta avhandlingsarbete har lett till en djupare förståelse för samspelet mellan fästingar, människor och borreliabakterier. Sådan kunskap kan bidra till att minska risken för fästingbett och borreliainfektioner hos människor.

List of papers

I. **Wilhelmsson, P.,** Lindblom, P., Fryland, L., Nyman, D., Jaenson, T.G.T., Forsberg, P., Lindgren, PE. *Ixodes ricinus* ticks removed from humans in Northern Europe: seasonal pattern of infestation, attachment sites and duration of feeding. *Parasit Vectors*. 2013 Dec; 6(1): 362.

II. **Wilhelmsson, P.,** Fryland, L., Börjesson, S., Nordgren, J., Bergström, S., Ernerudh, J., Forsberg, P., and Lindgren, PE. Prevalence and diversity of *Borrelia* species in ticks that have bitten humans in Sweden. *J Clin Microbiol*. 2010 Nov; 48(11): 4169-4176.

III. **Wilhelmsson, P.,** Lindblom, P., Fryland, L., Ernerudh, J., Forsberg, P., and Lindgren, PE. Prevalence, diversity, and load of *Borrelia* species in ticks that have fed on humans in regions of Sweden and Åland Islands, Finland with different Lyme borreliosis incidences. *PLoS One*. 2013 Nov; 8(11): e81433.

IV. **Wilhelmsson, P*.,** Fryland, L*., Lindblom, P., Sjöwall, J., Ahlm, C., Berglund, J., Haglund, M., Henningsson, AJ., Nolskog, P., Nordberg, M., Nyberg, C., Ornstein, K., Nyman, D., Ekerfelt, C., Forsberg, P., Lindgren, PE. A Prospective study on the incidence of *Borrelia* infection after a tick bite in Sweden and on the Åland Islands, Finland. *Manuscript.*
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Abbreviations

ACA	Acrodermatitis chronica atrophicans
BL	Borrelial lymphocytoma
bp	Base pair
cDNA	Complementary DNA
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
EM	Erythema migrans
FITC	Fluorescein isothiocyanate
kbp	Kilo base pair
LA	Lyme arthritis
LB	Lyme borreliosis
LC	Lyme carditis
IFA	Immunofluorescent antibody assay
Ig	Immunoglobulin
IGS	intergenic spacer
NB	Neuroborreliosis
Osps	Outer surface proteins
PCR	Polymerase chain reaction
PHC	Primary health care center
RNA	Ribonucleic acid
Salp	Tick salivary protein
TBD	Tick-Borne Disease
TBRF	Tick-Borne Relapsing Fever
TROSPA	Tick receptor for outer surface protein A
VlsE	Variable major protein-like sequence
WB	Western blot

Background

Lyme borreliosis (LB) is the most common tick-borne disease in the temperate regions of Europe, North America, and Asia with approximately 86,000 cases every year. LB is caused by a spirally shaped bacterium that belongs to the genus *Borrelia* and may manifest in different ways with mild to severe symptoms.

The causative agents of LB, i.e. members of the *Borrelia burgdorferi* senu lato complex, are transmitted to humans through the bite of infected ticks. A warmer climate with milder winters has in northern Europe lead to a gradual northward spread of such ticks. This expansion of ticks has resulted in an increased number of tick bites on humans and hence an increased number of LB cases. In Sweden as well as on the Åland Islands there is no LB notification system and thus, the incidence of LB is unknown. The overall aims of this thesis are to increase our understanding of the epidemiology of *Borrelia* bacteria, the ecology of ticks, as well as the clinical situation of LB in Sweden and on the Åland Islands. Furthermore, to provide information that can contribute reducing the risk for tick bites and the risk of developing LB after a tick bite.

Short review of the long history of Lyme borreliosis

The *Borrelia* spirochete has probably plagued mankind for thousands of years. An autopsy of a 5,300 year old Neolithic male mummy ("Ötzi"), discovered in the Italian part of the Ötztal Alps, revealed the presence of *Borrelia* DNA (Keller *et al.*, 2012). This makes "Ötzi" the earliest known human case of a *Borrelia* infection.

Description of a LB manifestation was first reported in the medical literature in 1883 (Buchwald, 1883). A 36 year old Polish bricklayer came to seek medical advice for a bluish red discoloration and cutaneous swelling on his left leg since 16 years back. The German physician Buchwald could not find the cause of the disease, and he described the symptoms as idiopathic skin atrophy. Later on, the disease was named acrodermatitis chronica atrophicans (ACA) (Herxheimer & Hartman, 1902).

In 1909, another unexplainable skin manifestation was described by the Swedish dermatologist Afzelius, erythema migrans (EM): a red skin lesion with expanding borders (Afzelius, 1910). During the following 12 years he encountered six EM cases, and proposed that the manifestation was caused by bites of insects or ticks (Afzelius 1921). By this time, a third skin manifestation with unknown cause was described as lymphocytoma (Burckhardt, 1911), a bluish-red pea-like nodule that appeared on earlobes, nipples or on scrotum (Bäfverstedt, 1943).

In France 1922, the two neurologists Garin and Bujadoux suspected a link between tick bites and neurological symptoms, such as facial palsy (Garin & Bujadoux, 1922). Twenty years later, the German neurologist Bannwarth noticed that some patients with neurological symptoms also had EM lesions (Bannwarth, 1941). By the end of 1940s, the Swedish dermatologist Lennhoff microscopically discovered spirochete-like elements in skin specimens taken from EM lesions (Lennhoff, 1948). He probably observed the etiology of EM – the *Borrelia* spirochete. Lennhoff's findings were not confirmed until 30 years later (Burgdorfer, 1984). But before that, in the 1950s, another Swedish dermatologist Hollström had showed that treatment with penicillin hasten the resolution of EM, which led to the use of

penicillin for treatment of such symptoms in several European countries (Hellerström, 1951; Hollström, 1951).

In 1970, the American dermatologist Scrimenti reported the first case of EM in the United States (i.e. Wisconsin). Based on the European literature, Scrimenti successfully treated the patient with penicillin (Scrimenti, 1970). Five years later, a mysterious outbreak of what originally appeared to be juvenile rheumatoid arthritis was reported among children from three towns in southeastern Connecticut, including the towns Lyme and Old Lyme. Due to the geographical origin of the outbreak, the disease was named Lyme arthritis. Dr. Steere and his colleagues suspected a link between the outbreak and tick bites (Steere *et al.*, 1977; Steere *et al.*, 1978). It also became clear that the disease could manifest in many various ways including neurologic, rheumatologic, dermatologic, and cardiac symptoms therefore the name Lyme arthritis was changed to Lyme disease. Intensive efforts were made to establish the cause of Lyme disease: acute and convalescent sera of patients were tested for antibodies against a numerous of viruses and bacteria. All tests were negative (Burgdorfer, 1984).

In 1981, Dr. Burgdorfer unexpectedly discovered a cluster of long, spirally shaped bacteria when he microscopically investigated the gut content of dissected ticks (Burgdorfer *et al.*, 1982). When Burgdorfer and his colleagues later on also discovered "identical" spirochetes in skin biopsies of EM patients, the missing link between tick bites and Lyme disease was finally found (Burgdorfer, 1984). Soon thereafter, reports indicated that similar spirochetes could cause other symptoms suggestive of Lyme disease as well (Asbrink *et al.*, 1984). The spirochete was named *Borrelia burgdorferi,* after its discoverer (Johnson *et al.*, 1984). In acknowledgment of this infectious agent, Lyme disease is today more often referred to as Lyme borreliosis.

The tick

Ticks are blood-feeding ectoparasites of mammals, birds and reptiles and more than 800 tick species have been found throughout the world (Barker & Murrell, 2004). They even existed when dinosaurs and primitive birds roamed the Earth. The oldest parasitiform fossil record of a tick was found in New Jersey and was dated back to the late Cretaceous more than 90 million years ago (Klompen & Grimaldi, 2001).

Ticks are arthropods, i.e. invertebrates with legs and joints, segmented body, and exoskeleton. They belong to the class of arachnids (Arachnida) together with spiders, scorpions and mites. Ticks are further classified into the subclass Acari, which include three families; Argasidae (soft ticks, 186 species)*, Ixodidae (hard ticks, 692 species)*, and Nuttalliellidae (monotypic) (Nava *et al.*, 2009). Hard ticks are generally distinguished from soft ticks by the presence of a hard shield that covers the dorsal region of the tick. Hard ticks also have a prominent capitulum (head with mouth and feeding parts) that projects forwards from the body; in soft ticks, the capitulum is located beneath the body. Both families comprise tick species that are important vectors of disease causing agents to humans and animals. These zoonotic agents are maintained in cycles between ticks and reservoir hosts, where humans can develop clinical illness but usually are "dead-end" hosts because they do not contribute to the transmission cycle. In Northern Europe, *Ixodes ricinus* is the most important hard tick vector to transmit disease-causing agents to humans; this includes bacteria, viruses, and parasites (Table 1). Co-infections of tick-borne pathogens in *I. ricinus* are commonly reported in the literature (Swanson *et al.*, 2006).

Table 1. Tick-borne pathogens transmitted by *Ixodes ricinus* ticks

Infectious organism	Disease	Reference
Anaplasma phagocytophilum (bacterium)	Human granulocytic anaplasmosis	Woldehiwet, 2010
Babesia divergens (protozoan)	Babesiosis	Vannier & Krause, 2012
Borrelia burgdorferi sensu lato (bacterium)	Lyme borreliosis	Stanek & Reiter, 2011
Borrelia miyamotoi (bacterium)	Tick-borne relapsing fever	Hovius *et al.,* 2013
Candidatus Neoehrlichia mikurensis (bacterium)	CNM-infection	Welinder-Olsson *et al.,* 2010
Francisella tularensis (bacterium)	Tularemia	Gurycova *et al.,* 1995
Rickettsia species (bacterium)	Rickettsioses	Raoult & Roux, 1997
Tick-borne encephalitis-virus (virus)	Tick-borne encephalitis	Floris *et al.,* 2006

Geographical distribution

The most important *Ixodes* tick species that transmit LB-causing spirochetes are *Ixodes ricinus* in Europe, *I. pacificus* in western North America, *I. scapularis* in eastern North America, and *I. persulcatus* in Asia (Figure 1). In Europe, *I. ricinus* can be found from the Faroe Islands in the west (Jaenson & Jensen, 2007) to the European section of the Russian Federation in the east (Korenberg *et al.*, 2002), and from North Africa (Zhioua *et al.*, 1999) to the Northern Scandinavia (Lindgren *et al.*, 2000).
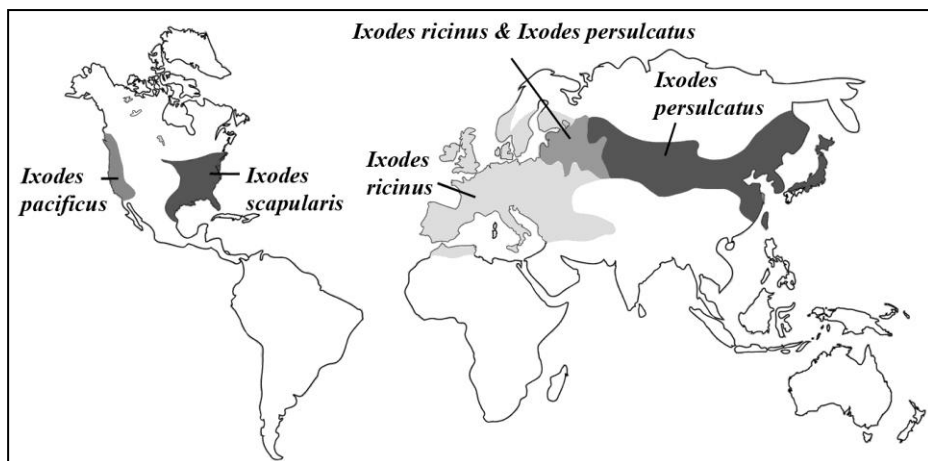
Figure 1. Distribution of important tick species (*Ixodes* spp.) able to transmit Lyme borreliosis-causing spirochetes to humans. (Courtesy of EUCALB, European Union Concerted Action on Lyme borreliosis, www.eucalb.com)

In Sweden, the geographical distribution of *I. ricinus* covers the southern and central parts of the country as well as the coastal area of northern Sweden. Jaenson and co-workers (Jaenson *et al.*, 2012) suggested that the climate change with milder winters and a prolonged vegetation period have permitted important *I. ricinus* maintenance hosts, particularly roe deer (*Capreolus capreolus*), to spread to and inhabit previously climatically, suboptimal areas in the northern parts of Sweden. This has resulted in a gradual spreading northwards of *I. ricinus* infesting deer; in this manner the range and abundance of *I. ricinus* in northern Sweden increased considerably during the last 30 years. This is has lead to an increased risk of tick bites and consequently an increased risk of tick-borne infections (Jaenson & Lindgren, 2011). On most islands of the Åland archipelago (Åland Islands), located between the mainlands of Sweden and Finland, tick bites are common, 85% of the inhabitants have sometimes been bitten by ticks (Wahlberg, 1990). The corresponding figure for the inhabitants of Sweden is unknown and data regarding tick bites on humans is poor. However, one telephone-based survey among 1,000

randomly selected Swedish residents (>15y/o) showed that one out of five (18%) experienced one or more tick bites during the tick season in 2005 (Boehringer-Ingelheim, 2006). This corresponds to more than 1.3 million tick bites in Sweden that year. To reduce the number of tick bites and tick-borne infections among people, it is important to raise public awareness about when during the year tick bites on people may occur, i.e. provide information regarding the tick seasonality.

Life of a tick

The *Ixodes* tick has three active life stages (larva, nymph and adult) (Figure 2). To develop from one stage to another, the tick has to feed on blood from a host.
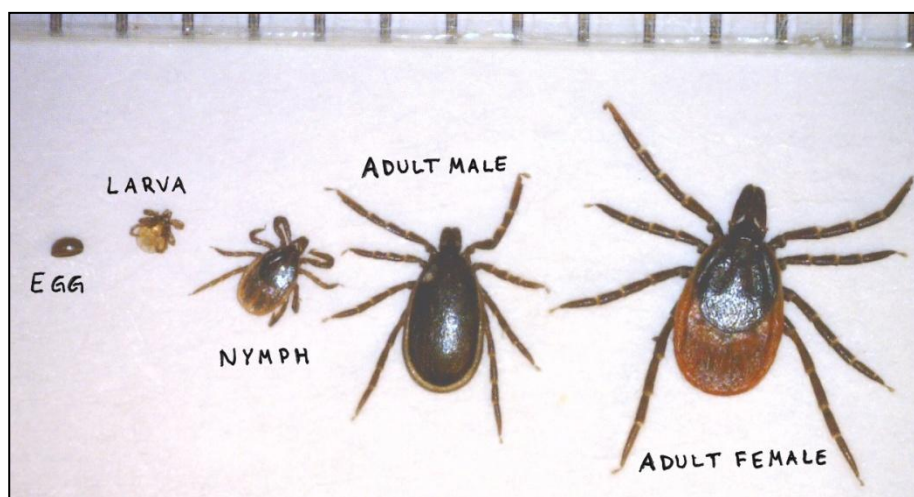

Figure 2. Life stages of *Ixodes ricinus*. Distance between two lines marked on the bar equals 1.0 mm. Photo: Peter Wilhelmsson

The six-legged larva, which emerges from the egg, crawls in grass and bushes and waits until a suitable host brushes against the vegetation.

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The *Ixodes* tick has no eyes but has a highly developed olfactory organ on its forelegs which gives warnings that a blood victim is approaching by detecting changes within the environment such as temperature, carbon dioxide, odours and vibrations (Suss, 2003). With the barb-like tarsi on its forelegs, the tick grasps and crawls up on the host. With its leg-like sensory palps, the tick searches for an area of thin, soft skin, ideal for inserting its feeding organ – the hypostome (Figure 3). Once a suitable spot has been found, the tick cuts the skin (epidermis and dermis) with its harpoon-like mouthparts and inserts its hypostome and starts to feed. In the salivary glands of the tick, various substances such as enzymes, vasodilators, anti-inflammatory substances, and anticoagulants are produced and injected directly into the host. This has a local anesthetic effect around the wound (Parola & Raoult, 2001). This may explain why one third up to two thirds of tick bites on humans may go unnoticed (Strle *et al.*, 1996; Strle *et al.*, 2002). Due to the backward projecting teeth of the tick, it remains attached to the skin until feeding is complete and it detaches (Anderson & Magnarelli, 1993). *Ixodes* ticks may feed 2-15 days and the duration is probably dependent on many factors, such as tick species, life stage, type of infested host, site of attachment among others (Parola & Raoult, 2001). For a feeding tick there is no risk of oxygen deficiency; oxygen diffuses from the air through the breathing holes (spiracles) of the tick, located behind the coxa of the fourth legs.

After a blood meal the fully fed larva drops to the ground and crawls to a place with high humidity. Here, it digests the blood meal and molts into the nymphal stage. After metamorphosis, the tick acquires an extra pair of legs. The eight-legged nymph searches for a new host to feed on before it can undergo the second metamorphosis and molt into sexual maturity in the adult stage (Parola & Raoult, 2001).

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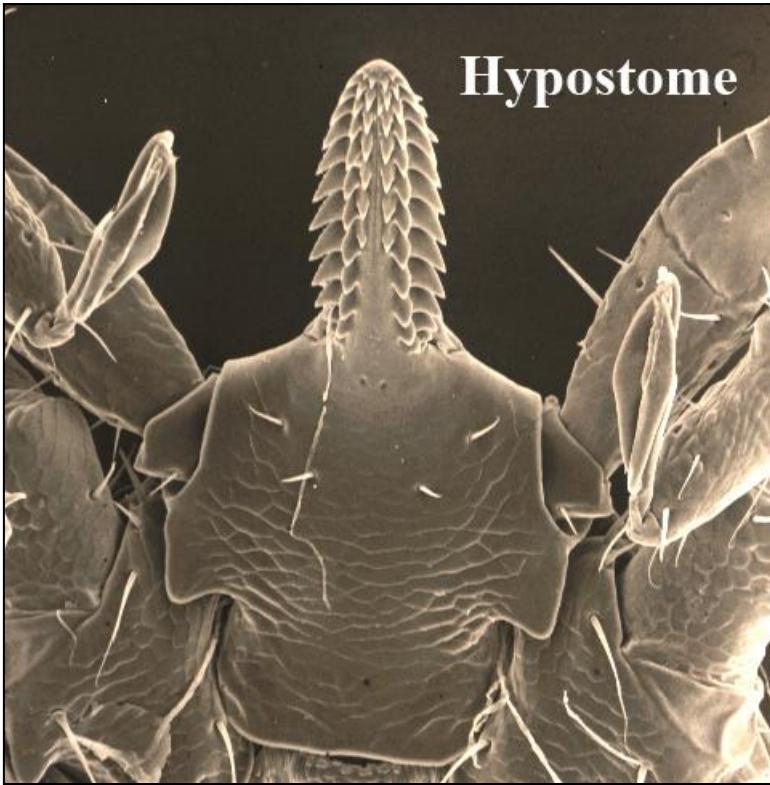
Figure 3. Scanning electron micrograph of the anterior, ventral part (basis capitulum) of a nymph of *Ixodes ricinus*. The median hypostome bears numerous recurved teeth, denticles, which are used for anchoring the feeding tick to the skin of the host. To the left and right sides of the hypostome are the movable palps discernible. Photo by Gary Wife & Thomas G.T. Jaenson©, Uppsala University. Permission to use image by Thomas G.T. Jaenson.

The adult female tick has two missions to accomplish before she can complete the life cycle: She has to copulate with an adult male tick, and she has to feed blood before she can produce eggs. The adult male tick, on the other hand, rarely feeds and never engorges. His main objective is to copulate with the female tick, a process that usually takes place on the host while the female tick is still feeding (Anderson & Magnarelli, 1993). The male tick delivers sperm cells by inserting his mouthparts into the genital pore of the female tick, which takes less than 1 h (Kiszewski *et al.*, 2001). After mating the

male tick dies, and the engorged, inseminated female tick drops to the ground where she, a few weeks later, lays several thousands of eggs and then dies. In a month, the eggs hatch into larvae that are ready to begin new quests for blood.

The whole life cycle usually takes 2-3 years to complete, but in cold climates, especially in northern latitudes where the number of suitable hosts may be limited, it may take up to 6 years (Anderson & Magnarelli, 1993). During such circumstances the tick may enter diapause, a condition characterized by reduced metabolism and postponed development (Parola & Raoult, 2001).

Temporal host-seeking behavior

The seasonal host-seeking activity pattern of *Ixodes* ticks is variable and not yet fully understood. However, it is influenced by several biotic and abiotic factors including vegetation type, density and variety of hosts, weather and climate, and photoperiod, which is dependent on latitude (Gray, 1991).

In two investigations conducted in south-central Sweden, nymphs and larvae of *I. ricinus* usually exhibited bimodal host-seeking activity patterns with the highest activity in May-June and in August-September (Mejlon & Jaenson, 1993; Talleklint & Jaenson, 1996). It is proposed that the midsummer activity depression in host-seeking activity of subadult ticks may partly be due to the relatively dry conditions that usually prevail at this time (Mejlon & Jaenson, 1993). During such a reduction in host-seeking activity, one would expect a lower tick infestation on animals and humans. In contrast to nymphs, adult ticks exhibited a unimodal host-seeking pattern without any midsummer depression (Mejlon & Jaenson, 1993).

Even if a tick is considered to be host-seeking, it is not evident that it would attach to and bite a human that passes by. The actual risk for people to get tick-bitten in tick-infested areas is dependent on the behavior of both ticks and humans, which in turn are influenced by weather conditions, climate and other factors. Information regarding such interaction could be used to increase the awareness of tick-borne infections in times when tick bites occur among people.

Spatial host-seeking behavior

Ixodes ticks are basically forest dwellers and become active when the temperature exceeds 4°C (Duffy & Campbell, 1994). They spend most of their time by hiding near the ground, where they are protected against sun light and desiccation. A longer exposure to dry air can be direct fatal to ticks (Rodgers *et al.*, 2007) and a relative humidity above 80% is necessary for tick survival (Gray, 1998). Larvae, which are particularly susceptible to desiccation due to their small body sizes, generally quest for hosts on the ground or on low vegetation where the humidity is higher (Mejlon & Jaenson, 1997). The nymphs also quest on the ground but also on vegetation one or a few decimeters above ground. In contrast to these low questing heights, the adult ticks often quest on vegetation 0.5-1.4 meters above ground. As a consequence, animals of different sizes serve as hosts for different life stages of the tick. Thus, larvae of *I. ricinus* feed mainly on small mammals such as rodents and shrews (Talleklint & Jaenson, 1997) and on small ground-frequenting birds (Olsen *et al.*, 1995), whereas nymphs and adult ticks usually infest medium-sized and larger mammals, e.g. hares and roe deer (Talleklint & Jaenson, 1997).

Attachment sites on animals

The different questing heights of the tick stages may partly influence their different attachment locations (sites) on their hosts. On the European roe deer, *Capreolus capreolus*, larvae, nymphs and adult females of *I. ricinus* show high degrees of interstadial aggregation (Kiffner *et al.*, 2011): Larvae aggregate mainly to the forelegs and to the head of roe deer, nymphs aggregate mainly to the head, and adult females aggregate mainly to the neck of roe deer. On sheep, larvae of *I. ricinus* attach mainly to the lower parts of the body and adult females mainly to the higher parts, while nymphs will mainly attach to sites in between those of larvae and adults (Ogden *et al.*, 1998). Tick-stage related "preferences" for site of attachment have also been observed for the American *I. scapularis* ticks on the white tailed deer, *Odocoileus virginianus*: Adult ticks attached mainly on the anterior dorsal body regions, 87% of adult ticks attached to the outside of the ears, head, neck and brisket (Schmidtmann *et al.*, 1998). However, on horses, attachment by adult female *I. scapularis* was largely restricted to the under-body areas, which was considered to reflect avoidance of direct sunlight by the ticks.

Stage-specific degrees of tolerance of desiccation may be one among factors, which explain how stage-specific "preferences" for attachment sites have evolved. However, the grooming behavior of the host and the capacity to remove ectoparasites from particular parts of the body of the host should have a pronounced effect on the evolution of feeding sites "preferred" by ticks.

Clinical importance of attachment site and duration of tick feeding on humans

The site of tick attachment on the human body may be of clinical importance. Berglund and co-workers carried out an extensive epidemiological study of LB among 1471 LB patients in southernmost Sweden (Berglund *et al.*, 1995). They recorded a significantly higher proportion (20%) of neurological manifestations among LB patients bitten by ticks (probably caused by *I. ricinus*) on head and neck, than among patients bitten on other parts (7%). Berglund and co-workers also recorded that 49% of the bites in children (≤15 years) were located in the head and neck region, as compared with 2% among the adults. This could suggest fundamental differences in how ticks respond to hosts of different sizes and/or ages. Therefore, information about where on the human body *I. ricinus* ticks usually attach, could be used to increase the effectiveness of prophylactic actions to reduce the risk for tick bites. This may include development of protective clothing, where on the human body tick repellents should be used, and where on the human body one should check for ticks.

The duration of tick attachment has been closely associated with the efficacy of *Borrelia* spirochete transmission from tick to laboratory animals (Crippa *et al.*, 2002; Kahl *et al.*, 1998; Piesman *et al.*, 1987): The longer a *Borrelia*-infected tick remains attached to the skin, the greater the risk of contracting a *Borrelia*-infection. This is probably also the case for tick-bitten humans. Prompt removal of attached ticks is therefore a prudent public health measure. However, when ticks attach to certain "hidden" areas on the human body it may be more difficult to detect the tick. *Ixodes* ticks that attach to the head and neck area of people are usually detected and removed later than ticks attached to other parts of the body (Falco *et al.*, 1996; Hugli *et al.*, 2009). Information about the duration of tick attachment, i.e. the

time it takes for tick-bitten person to discover and remove the tick, could therefore be used to assess the likelihood that a *Borrelia* infection will take place and to judge the risk that LB symptoms will be developed.

For *I. scapularis* nymphs, the duration of attachment appears to increase with the age of the bitten person (Falco *et al.*, 1996): A significantly higher proportion (52%, 16/31) of persons between 50 and 59 years had nymphs attached for more than 48 hours, compared with the proportion of children under the age of 10 years (19%, 34/182). This indicates that *Borrelia* transmissions are more likely to occur among older people bitten by *Borrelia*-infected *I. scapularis* nymphs compared to younger people. In contrast, a higher proportion (37%, 39/105) of children under age 10 years had *I. scapularis* adult females attached for more than 48 hours compared with the proportion of tick-bitten persons between 50 and 59 years (21%, 6/28). This indicates that *Borrelia* transmission is more likely to occur among younger people bitten by *I. scapularis* adult females compared to older ones. All this together suggests that persons under the age of 10 years and persons older than 50 years are at special risk of contracting LB when they are bitten by *Borrelia*-infected ticks. In support, the age distribution of the LB disease in most countries is usually bimodal with the first (lower) maximum occurring in children 5-9 years old, and the second (higher) maximum in persons 50-64 years old (Hubalek, 2009).

The *Borrelia* spirochete

The causative agent of LB is a spirally shaped type of bacterium that is called a spirochete and belongs to the phylum Spirochaetae, which consists of three families: Brachyspiraceae, Leptospiraceae and Spirochaetaceae. The group of LB-causing spirochetes, *Borrelia burgdorferi* sensu lato, is classified into the latter family (Paster & Dewhirst, 2000). The *B. burgdorferi* sensu lato spirochetes possess several morphological, structural, genomic and other features that are distinctive among bacteria.

Morphological and structural features

The *Borrelia* bacterium is one of the largest of the spirochetes, 10-30 µm in length, and < 1 µm in width (Barbour & Hayes, 1986). It has a flat-waved shaped body configured with 3 to 10 loose coils and an internal arrangement of 7 to 11 endoflagella bundled together in its periplasmic space between the inner and outer membrane (Figure 4). The flagella run the length of the spirochete from tip to tip and when they rotate, the spirochete contracts like a large muscle which causes it to move either forward or backward in a corkscrew fashion. This makes the *Borrelia* spirochete to a highly motile bacterium and allows it to efficiently swim through blood and tissues.
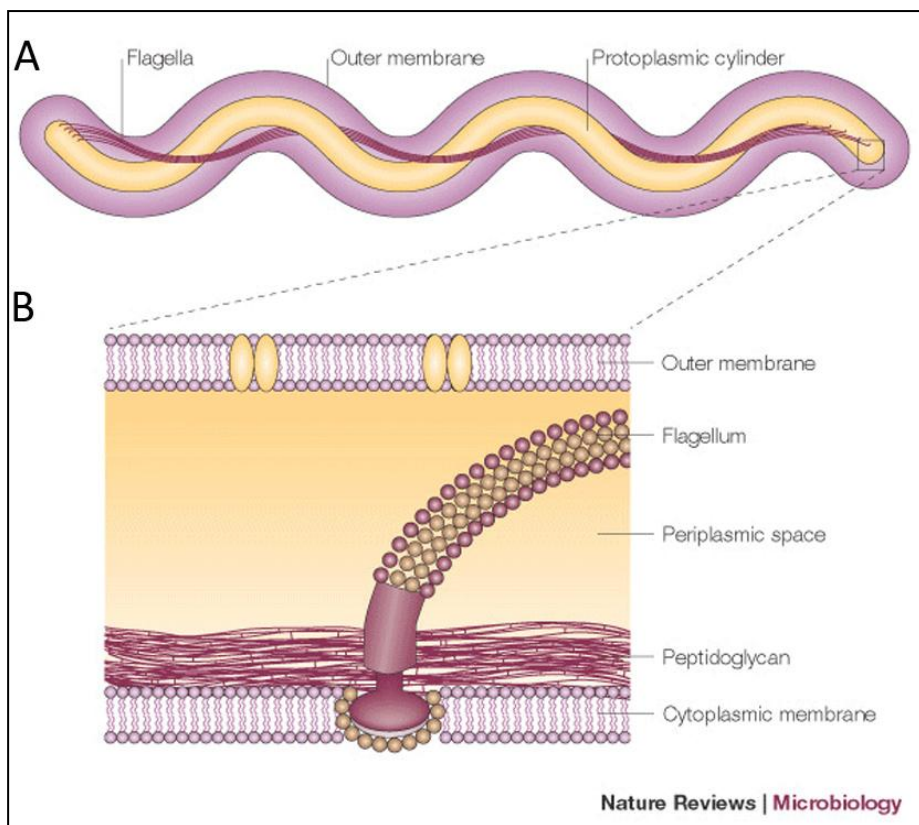
Figure 4. Schematic illustration of *Borrelia burgdorferi* sensu lato (A) and of its cell wall (B). (Reprinted from The burgeoning molecular genetics of the Lyme disease spirochaete, (Rosa *et al.*, 2005), Nature Reviews Microbiology, Copyright 2005 with permission from Nature Publishing Group).

The *Borrelia* spirochete is described as a Gram-negative bacterium. However, what makes this bacteria different from other Gram-negative species is that it lacks the presence of lipopolysaccharides on its outer membrane (Takayama *et al.*, 1987). Instead, the spirochete is coated with lipoproteins called outer surface lipoproteins (Osps) (Luft *et al.*, 1989). The Osps seem to play important roles in dissemination and in immune evasion of the bacteria (Kenedy *et al.*, 2012). They may act as receptors for various molecules and targets for bactericidal antibodies (Wilske *et al.*, 1992). Depending on the

surrounding environment, the *Borrelia* bacteria may alter the expression of the Osps (Rupprecht *et al.*, 2008). This means that a lipoprotein that has become the target for antibodies can be down-regulated, thus making the spirochete "invisible" for the immune system.

Reproductive and genomic features

The *Borrelia* spirochete is a microaerophilic bacterium and has an obligate parasitic lifestyle, which means it cannot live outside the body of a tick or a host. The *Borrelia* spirochete multiply and reproduce by transverse binary fission, where the division is preceded by a longitudinal growth of the individual spirochete (Fritzsche, 2005). This process is slow and takes between 12 and 24 hours during log-phase growth *in vitro* (Barbour, 1984).

All species of the *B. burgdorferi* sensu lato group that have been genetically investigated, harbor a genome that consists of a linear chromosome (~ 910 kbp in length) and up to 21 extrachromosomal DNA elements (~ 600 kbp); 12 linear and 9 circular plasmids (Casjens, 2000). This is the largest number of plasmids known for any bacterium (Casjens *et al.*, 2000). The plasmids, which are highly variable across the genus, carry most of the genes that encode the differentially expressed outer surface lipoproteins. The chromosome, which is highly conserved across the *Borrelia* genus, carries the vast majority of the genes that encode metabolic enzymes and it also carries components of the ribosome, rRNA genes, which are essential for protein synthesis. The absence of genes for the synthesis of amino acids, fatty acids, enzyme cofactors, and nucleotides suggests that many nutritional components are instead provided by the host (Fraser *et al.*, 1997). This is probably the reason why the *Borrelia* bac-

terium requires an extremely complex medium when it is cultivated *in vitro*.

Other features

Bleb and cyst formations are another unique features of some *Borrelia* spirochetes. When the spirochetes undergo different stress conditions *in vitro*, e.g. contact with penicillin or specific antibodies, starvation due to prolonged cultivation, or if they are freeze-thawed, they start to replicate specific plasmid genes, e.g. DNA that encodes surface lipoproteins, and inserts them into its own cell wall (Garon *et al.*, 1989; Persing *et al.*, 1994). Those parts of the cell wall are then pinched off as extracellular vesicles, blebs, which are coated with surface lipoproteins and contain plasmid DNA. The reason for this is unknown but in other bacteria, e.g. *Neisseria gonorrhoeae*, the appearance of blebs can constitute genetic exchange between bacteria populations (Dorward *et al.*, 1989).

According to some researchers, the *Borrelia* bacterium may also undergo cyst formation when it is under similar stress conditions as for the bleb formation (Miklossy *et al.*, 2008). These small (Ø 0.5-2 μm) cysts may, if the stress stimuli are removed, regenerate back into full size spirochetes and reproduce (Brorson & Brorson, 1997; Brorson & Brorson, 1998). This low-active state of the bacteria may be important for their survival in a non-favorable environment.

Borrelia-infected ticks

Worldwide distribution of *Borrelia* species in *Ixodes* ticks

The genus *Borrelia* comprises many species. The *B. burgdorferi* sensu lato complex is a group of spirochetes that may cause human LB. This complex includes 18 named *Borrelia* species which are present in *Ixodes* ticks that can be found in the temperate regions of Europe, North Africa, Asia, and North America (Table 2). Descriptions of new *Borrelia* species are continuously recognized, so the current number of described species of the *B. burgdorferi* sensu lato complex is probably not final (Stanek & Reiter, 2011).

Besides the species of the *B. burgdorferi* sensu lato complex, another group of *Borrelia* species, the tick-borne relapsing fever (TBRF) species, are also pathogenic to humans. They may cause a disease that is characterized by influenza-like illness and recurring episodes of fever. Today, at least 15 different TBRF species have been identified (Ras *et al.*, 1996; Rebaudet & Parola, 2006) and the disease is reported in North and South America, Africa, Asia and Europe, where they are transmitted to humans mainly by soft ticks of the genus Ornithodoros. However, one of the TBRF species, *B. miyamotoi*, has been found in a small percentage of *Ixodes* ticks. This species was first discovered in *I. persulcatus* ticks in Asia (Fukunaga & Koreki, 1995), and it has also been found in *I. scapularis* and *I. pacificus* in North America (Bunikis *et al.*, 2004; Scoles *et al.*, 2001) and *I. ricinus* in Europe (Bunikis *et al.*, 2004; Fraenkel *et al.*, 2002).

Besides the LB-causing and TBRF-causing species, other *Borrelia* species such as *B. anserina* and *B. coriaceae* have been found to be the etiological agents of avian and bovine spirochetosis, respectively (LeFebvre & Perng, 1989; McNeil *et al.*, 1949).

Table 2. The *Borrelia burgdorferi* sensu lato complex, its tick vectors, and its geographical distribution

Borrelia species	Main tick vector	Distribution
B. afzelii [1]	*I. ricinus, I. persulcatus*	Europe, Asia
B. garinii [1]	*I. ricinus, I. persulcatus, I. uriae*	Europe, Asia
B. burgdorferi sensu stricto [1]	*I. ricinus, I. scapularis, I. pacificus*	Europe, North America
B. lusitaniae [2]	*I. ricinus*	Europe, North Africa
B. spielmanii [2]	*I. ricinus*	Europe
B. valaisiana [2]	*I. ricinus, I. columnae*	Europe, Asia
B. bavariensis [2]	*I. ricinus*	Europe
B. bissettii [2]	*I. ricinus, I. scapularis, I. pacificus*	Europe, North America
B. americana [3]	*I. pacificus, I. minor*	North America
B. andersonii [3]	*I. dentatus*	North America
B. californiensis [3]	*I. pacificus*	North America
B. carolinensis [3]	*I. minor*	North America
B. kurtenbachii [3]	*I. scapularis*	North America
B. japonica [3]	*I. ovatus*	Asia
B. sinica [3]	*I. ovatus*	Asia
B. tanukii [3]	*I. tanuki*	Asia
B. turdi [3]	*I. turdus*	Asia
B. yangtze [3]	*I. granulatus*	Asia

[1] Human pathogen, [2] suspected/potential pathogen, [3] unknown human pathogenicity. The distribution of *B. burgdorferi* species, their human pathogenicity and their tick vectors are reviewed by Stanek & Reiter, 2011.

European distribution of *Borrelia* species in *Ixodes ricinus*

The prevalence and distribution of *Borrelia* species in ticks are some of the most essential components of risk assessment for LB and have therefore been extensively studied in Europe. The results from a meta-analysis based on more than 112,500 host-seeking *I. ricinus* ticks collected from 24 European countries between 1984 and 2003, showed an overall mean *B. burgdorferi* sensu lato prevalence of 14% (Rauter & Hartung, 2005). However, the prevalence was heterogeneously distributed among the studied regions of Europe (Figure 5). In general, a higher prevalence was usually found in regions located in central Europe, but also in regions located in northern Europe as well as in regions located in southern Europe. Lower prevalence of *Borrelia*-infected ticks was found in the surrounding regions.

A higher proportion of adult ticks (19%) were infected with *B. burgdorferi* sensu lato compared to nymphs (10%) (Rauter & Hartung, 2005). This is probably related to the higher number of blood meals ingested by the adult ticks. The most common *Borrelia* species that was found was *B. afzelii* (55%), followed by *B. garinii* (21%), *B. burgdorferi sensu stricto* (10%), *B. valaisiana* (9%), *B. lusitaniae* (1%), and untypeable species (4%). Other studies have also reported the detection of *B. spielmanii*, *B. bissettii*, *B. bavariensis* and the TBRF-causing species *B. miyamotoi* in a small percentage of *I. ricinus* ticks collected from regions in Europe (Bunikis *et al.*, 2004; Fraenkel *et al.*, 2002; Hanincova *et al.*, 2003b; Perez *et al.*, 2012; Richter *et al.*, 2004).
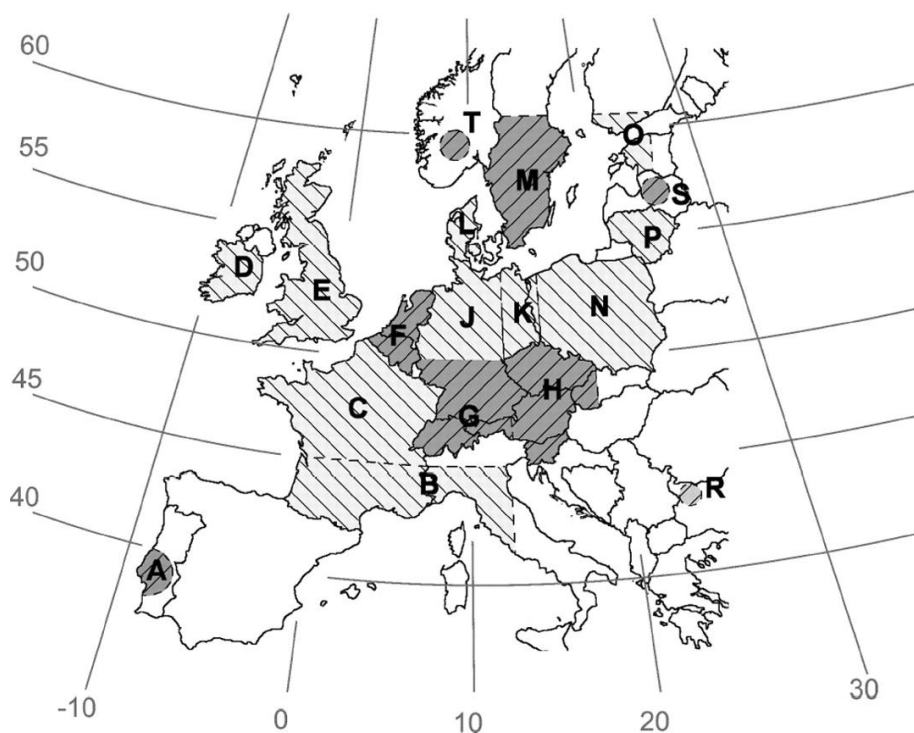
Figure 5. Prevalence of *Borrelia*-infected *Ixodes ricinus* in Europe. Areas with low infection rates (nymphs, ≤11%; adults, ≤20%) are indicated by light gray; areas with higher infection rates are indicated by dark gray. Areas not included in the metaanalysis are indicated by white (Reprinted from Prevalence of *Borrelia burgdorferi* sensu lato genospecies in *Ixodes ricinus* ticks in Europe: a metaanalysis, (Rauter & Hartung, 2005), Applied and Environmental Microbiology 71, Copyright 2005 with permission from American Society for Microbiology).

The prevalence of some *Borrelia* species may vary widely in different regions of Europe (Rauter & Hartung, 2005). For instance, significantly more ticks collected from Northern Europe (Norway, Sweden, Finland, and Estonia) and from central Europe (southern Germany, Czech Republic, Slovakia, Bulgaria, Croatia, and Slovenia) were infected with *B. afzelii* than with *B. garinii*. In contrast, *B. garinii* predominated among ticks collected from United Kingdom, Ireland and central and northern Germany. In Austria, Switzerland, the Netherlands,

Belgium and northern France, no significant difference between the prevalence of *B. afzelii* and *B. garinii* in the collected ticks were found. Furthermore, 70% of all *B. lusitaniae* species presented in the meta-analysis was detected in *I. ricinus* ticks collected from Portugal (Rauter & Hartung, 2005).

The prevalence of *Borrelia* species may also vary between the stages of *I. ricinus*. In general, *B. afzelii* was more prevalent in nymphs than in adult ticks and *B. garinii* was more prevalent in adult ticks than in nymphs (Rauter & Hartung, 2005). Tick stage-specific differences in the prevalence of *B. burgdorferi* sensu lato species might be due to the prevalence of different reservoir hosts. *B. afzelii* has frequently been associated with rodent populations (Hanincova *et al.*, 2003a; Kurtenbach *et al.*, 2002b), *B. garinii* and *B. valaisiana* have been linked to avian populations (Hanincova *et al.*, 2003b), *B. burgdorferi sensu stricto* is a species that has the ability to persist in a wide range of vertebrates (Kurtenbach *et al.*, 2002a), and *B. lusitaniae* has been associated with lizard populations (Dsouli *et al.*, 2006; Richter & Matuschka, 2006).

In the meta-analyses, 13% of all the analyzed *I. ricinus* ticks (adult ticks 14%, nymphs 12%) had a mixed infection, i.e. more than one *Borrelia* species per tick (Rauter & Hartung, 2005). Of these, 96% had a double infection, where the most frequent combination of *Borrelia* species was *B. garinii* and *B. valaisiana*. Combinations of three or more species only rarely occurred. Mixed infections could be explained by co-transmission of multiple *Borrelia* species from an infected tick to an uninfected tick feeding on the same host, or by co-transmission of several strains from a host infected by more than one *Borrelia* species, or by consecutive infectious blood meals (Rauter & Hartung, 2005). Theoretically, since adult ticks compared to nymphs have fed from two potentially *Borrelia*-infected hosts, they should

have a higher prevalence of mixed infections. However, this was not the case, adult ticks and nymphs had a similar prevalence of mixed infections (14% and 12%, respectively). The explanation of the authors for this is that the *Borrelia* species, obtained from the first blood meal, may be eliminated in the midgut of the tick due to the effect of complement taken up during the second blood meal (Rauter & Hartung, 2005). In support, complement-mediated borreliacidal effects have been observed with particular combinations of host serum and *Borrelia* species (Kurtenbach *et al.*, 1998). This could potentially lead to clearance of an already existing *Borrelia* infection in the tick.

No information regarding the prevalence of *B. burgdorferi* sensu lato in larvae was presented in the meta-analyses (Rauter & Hartung, 2005). However, the larva stage is considered to be a less important source of *Borrelia* infection for humans; it is almost never found to be infected with LB-causing spirochetes (Richter *et al.*, 2012). Therefore, it is presumed that transovarial transmission of *B. burgdorferi* sensu lato in *I. ricinus* rarely, if ever, takes place (Rollend *et al.*, 2013). But a small proportion of larval *I. ricinus* may be natural vector of the TBRF-causing agent *B. miyamotoi* (Richter *et al.*, 2012; Rollend *et al.*, 2013). Transovarial transmission of spirochetes in *I. ricinus*, previously thought to belong to *B. burgdorferi* sensu lato, needs confirmation. It is most likely that *B. miyamotoi* is responsible for these earlier reports of transovarial transmission of the bacteria by *I. ricinus* just as by *I. scapularis* (Rollend *et al.*, 2013).

As stated in the beginning of this chapter, the prevalence and distribution of *Borrelia* species in ticks are some of the most essential components of risk assessment for LB. Such information is also valuable when diagnostic tools are developed. Most of our knowledge of prevalence of *Borrelia* species in ticks originates from studies on ticks

from vegetation or ticks from animals. To obtain a deeper knowledge and understanding of the epidemiology of the *Borrelia* bacteria and the LB disease, it is important to also investigate the prevalence and distribution of *Borrelia* species in ticks that have actually bitten humans.

Transmission routes of *Borrelia* spirochetes

From host to tick

A tick acquires a *Borrelia* infection primarily through feeding on an infected reservoir host, which is defined as a vertebrate host animal that is capable of passing *Borrelia* spirochetes to a feeding tick vector. *I. ricinus*, the predominant *Ixodes* species in Europe, may feed on more than 200 animal species, including mammals, birds, and reptiles (Gern, 2008), where the *Borrelia* reservoir potential varying between animal species. During tick feeding, the *Borrelia* spirochetes will be engorged with the blood and finally end up in the tick gut. Here, the *Borrelia* spirochete up-regulates the expression of an outer surface protein called OspA, which it uses to anchor itself to the gut epithelium via the tick receptor for OspA (TROSPA) (Pal *et al.*, 2004). After the subsequent molting of the tick, there is an approximately 10-fold drop in *Borrelia* spirochete concentration (Piesman *et al.*, 1990), which suggests that some spirochetes are digested during the blood meal. The remaining spirochetes may multiply by time but have to pass the time in the tick gut for months or years until the tick feeds again; a siege that may involve extreme temperature fluctuations caused by seasonal changes.

From tick to host

When the *Borrelia*-infected tick takes a new blood meal from a host, the ingested blood changes the environment of the tick gut with regard to temperature, pH and nutrient levels. Together, these changes trigger the *Borrelia* spirochete migration. The spirochetes down-regulate their OspA expression and detach from the TROSPA receptors in the tick gut epithelium (Ohnishi *et al.*, 2001). Instead, OspC expression is up-regulated (Schwan *et al.*, 1995), and the spirochetes start to cross the gut epithelial barrier and disseminate to the haemocoel for transmission to the salivary glands (Coleman *et al.*, 1997). In the salivary glands, OspC binds to a tick salivary protein (Salp15) (Ramamoorthi *et al.*, 2005), a protein that appears to have immunosuppressive effects, enhancing infection of the host (Anguita *et al.*, 2002; Garg *et al.*, 2006). From the salivary glands the spirochetes disseminate into the host.

Piesman and coworkers reported that the number of spirochetes (*B. burgdorferi sensu stricto*) in the guts of feeding *I. scapularis* nymphs increased sixfold, from a total of 998 per tick to 5,884, during the first 2 days of feeding on mice (Piesman *et al.*, 2001). The full process of spirochete-migration from tick to a host can be as short as 17 hours of tick feeding (Kahl *et al.*, 1998) but a particularly efficient transmission takes place after 72 hours (Piesman *et al.*, 1987). The time it takes for a spirochete to be transmitted from a tick to a host is probably dependent on many factors such as tick species, life stage of the tick, *Borrelia* species, initial number of *Borrelia* cells in the tick, and type of host. Little is known about the process of spirochete-migration from tick to human. By studying how the number of *Borrelia* cells in a tick that feeds on a human is affected by the duration of tick feeding and simultaneously study the serological response

in humans bitten by such ticks, could contribute to a deeper under-standing of the *Borrelia* transmission from tick to human.

From tick to tick

A spirochetal transmission from tick to tick can take place via a direct passage between co-feeding ticks. Co-feeding ticks constitute an ag-gregation of ticks feeding on the same host. In this way, the *Borrelia* bacteria can be spread between ticks without systemically infecting the host first. Spirochetes remain at the site of deposition in the skin of the animal for a few days before disseminating into the host (Shih *et al.*, 1992), which allows ticks to become infected from a localized infection. The success of co-feeding transmission is probably influ-enced by the duration of feeding and the distance from the infecting tick (Richter *et al.*, 2002), the closer they are and the longer time they feed the likelihood of transmission increases.

Although some *Borrelia* species that cause TBRF may be readily passed from adult female to egg via transovarial transmission, this appears to be an exceptionally rare event for *B. burgdorferi* sensu lato if not impossible (Richter *et al.*, 2012; Rollend *et al.*, 2013). This suggests that ticks in the larval stage are not an important source of LB infection for humans.

Host response and immune evasion of *Borrelia*

The *Borrelia* spirochete has been isolated from several organs, tissues and body fluids of LB patients; it can be deeply embedded inside the skin, the heart, the brain (Stanek *et al.*, 2011), and even in the eye (Preac-Mursic *et al.*, 1993). To disseminate from the site of the tick bite, the spirochete must swim through connective tissue, blood vessel walls, extracellular matrix, and then back through blood vessel walls, and finally into the target tissue itself, concurrently as it must evade the host immune response. This makes one wonder how the spirochete gets around in the human body.

Innate immune evasion strategies

As soon as a tick pierces the layer of a skin, damaged skin cells release chemical messengers which cause vasodilatation where red and white blood cells are collected. The tick saliva, which contain enzymes, vasodilators, anticoagulants, and anti-inflammatory substances, is injected into the skin during tick feeding (Parola & Raoult, 2001). This creates a pit of pharmacologically active substances that impair homeostasis and wound healing (Nuttall & Labuda, 2004). After many hours of tick feeding, the *Borrelia* spirochetes begin to bump into the pit. Here, they may remain locally for days, which may represent an adaptation to the new environment (Shih *et al.*, 1992). Meanwhile, the *Borrelia* spirochetes encounter the first contact with the host's innate immune system which includes: engulfment of phagocytic cells, induction of pro-inflammatory proteins, and complement-mediated lysis (Steere *et al.*, 2004).

The complement system enhances the ability of antibodies and phagocytic cells to clear pathogens from an organism. To avoid dam-

age to normal cells, the activation of the complement system is strict-ly regulated by several factors, for example, factor H. The *Borrelia* bacteria have evolved mechanisms for recruiting factor H which pro-vides significant resistance to complement attack, and therefore in-creased virulence (Alitalo *et al.*, 2002; Connolly & Benach, 2005; Stevenson *et al.*, 2002).

Dissemination and colonization

Borrelia spirochetes that survive the first contact with the innate im-mune cells of the host can begin to disseminate in the body. Even if the *Borrelia* spirochete lacks the ability to produce proteases, it can bind host proteases to assist in dissemination and penetration into host tissue. Borrelial binding and activation of host plasminogen, an enzyme capable of degrading extracellular matrix, has been demon-strated *in vitro* (Coleman *et al.*, 1995; Grab *et al.*, 2005; Hu *et al.*, 1995; Klempner *et al.*, 1995). Activated plasminogen (plasmin) at-tracts inflammatory cells that induce enzymatic reactions which can dissolve cell membranes, connective tissue, and tendons. This allows the *Borrelia* spirochete to penetrate virtually any tissue of the human body. The *Borrelia* spirochete is also a highly motile organism having several periplasmic flagella that facilitate its spread to target sites and subsequent penetration of tissue barriers (Li *et al.*, 2000).

Host cell adherence of the *Borrelia* spirochetes is an initial step for host colonization. The *Borrelia* spirochetes have been shown to ad-here to human and murine fibroblasts, endothelial cells, epithelial cells, macrophages, neuronal and glial cells, fibrocytes, and lympho-cytes (Chmielewski & Tylewska-Wierzbanowska, 2010; Cinco *et al.*, 2001; Coburn *et al.*, 1998; Dorward *et al.*, 1997; Fischer *et al.*, 2003; Grab *et al.*, 1999; Leong *et al.*, 1998; Livengood & Gilmore, 2006;

Montgomery *et al.*, 1993; Peters & Benach, 1997; Rupprecht *et al.*, 2006; Sambri *et al.*, 1993; Thomas *et al.*, 1994). Although the *Borrelia* spirochete is generally referred to as an extracellular pathogen, several investigations performed *in vitro* with cell cultures have demonstrated the invasive properties of *Borrelia* bacteria to non-professional phagocytic cells (Chmielewski & Tylewska-Wierzbanowska, 2010; Girschick *et al.*, 1996; Hechemy *et al.*, 1992; Klempner *et al.*, 1993; Livengood & Gilmore, 2006; Ma *et al.*, 1991; Wu *et al.*, 2011). Another *in vitro* study showed that the *Borrelia* spirochete actively attaches to, invades and kills human T-cells and antibody-producing B-cells (Dorward *et al.*, 1997). The demonstration of *Borrelia* spirochetes within cells suggests that intracellular localization may be a potential mechanism by which the organism escapes from the immune response of the host. However, it is still unclear whether or not the *Borrelia* spirochetes invade cells during natural infections.

Adaptive immune evasion strategies

Activated B-cells may produce IgM antibodies within the first weeks of a *Borrelia* infection (Aberer & Schwantzer, 2012). IgM antibodies bind to borrelial antigens and mark them for destruction by immune cells. In order for the immune system to make an attacking antibody, the immune system must first find an antigen which it can attack. In the initial phase of a *Borrelia* infection, the *Borrelia* spirochetes express a lipoprotein (OspC) on their surfaces which are coated with Salp15. Salp15 has been shown to inhibit helper T-cell activation (Anguita *et al.*, 2002), potentially by masking the exposure of OspC to components of the host immune system, thus enhancing *Borrelia* survival and infection in the host. Interestingly, when a *Borrelia* spi-

rochete is under the influence of immune pressure, its expression of OspC is down-regulated and instead expressions of other surface lipoproteins are up-regulated (Liang *et al.*, 2004). One of those proteins, called variable major protein-like sequence (VlsE), has variable regions that masks the presence of *Borrelia* spirochetes from the immune system and allow it to escape (Liang *et al.*, 2000). When the VlsE is synthesized, the *Borrelia* spirochete periodically replaces the variable regions with new sequences. This replacement presents fresh surface antigens and helps the spirochete remain "invisible" to components of the specific immune response such as antibodies.

Clinical manifestations and diagnosis of Lyme borreliosis

Lyme borreliosis is an infectious disease which may affect several organs and tissues of the human body (Stanek *et al.*, 2011). The skin, joints and nervous system are the most affected and the symptoms can be absent or mild to some individuals, and devastating to others. Differences in clinical manifestations between LB patients in Europe and North America are well documented (Nadelman & Wormser, 1998; Steere, 2001; Wang *et al.*, 1999b). Such differences are attributed to differences in *B. burgdorferi* sensu lato species, causing LB on both sides of the Atlantic. In North America, *B. burgdorferi sensu stricto* is the only species known to be human pathogenic whereas in Europe eight different species have been associated with clinical manifestation of LB (Table 2).

Erythema migrans
Erythema migrans (EM) is the most identifiable early symptom of LB, which may appear days to weeks after the tick bite. It is characterized

by a red skin lesion that may enlarge from the site of the tick bite to five cm or greater, and sometimes a central clearing develops and form the classic "bulls-eye lesion" (Stanek & Strle, 2003). EM affects all ages and both sexes and is recognized in around 80% of LB patients in Europe and North America (Berglund *et al.*, 1995; Huppertz *et al.*, 1999; Mehnert & Krause, 2005). The patient often experience flu-like symptoms, such as fatigue, arthralgias, myalgias, fever and headaches (Steere, 2001). Multiple EM may also occur but are usually not the result of multiple tick bites; it rather indicates disseminated infection of the *Borrelia* spirochetes (Bratton *et al.*, 2008). The diagnosis of EM is rather clinical than serological, patients with typical lesions are usually seronegative for *Borrelia* (Stanek *et al.*, 1996; Strle, 1999). Characterization of *Borrelia* spirochetes in skin isolates taken from European patients revealed that EM is most often caused by *B. afzelii* (74-94%), less frequently by *B. garinii* (6-26%) and rarely by *B. burgdorferi sensu stricto*, *B. bissettii*, *B. valaisiana*, and *B. spielmanii* (Bennet *et al.*, 2006; Cerar *et al.*, 2008; Foldvari *et al.*, 2005; Hulinska *et al.*, 2009; Ornstein *et al.*, 2001; Strle & Stanek, 2009).

Borrelial lymphocytoma

Borrelial lymphocytoma (BL) is presented as a single bluish-red nodule and is typically located on the earlobe in children and near the nipple or scrotum in adults, usually close to the area of the tick bite (Strle *et al.*, 1992). The nodule consists of a dense lymphocytic infiltration as a result of *Borrelia* infection and its regional specificity suggests that the BL-causing spirochetes prefer lower host body temperatures (Vasudevan & Chatterjee, 2013). Patients may also present with BL and a simultaneous or precedent EM (Strle *et al.*, 1992). Diagnosis of BL is based on the clinical picture and may be supported by

positive serology, histological examination, and/or polymerase chain reaction (PCR) analysis of biopsies (Colli *et al.*, 2004). Characterization of *Borrelia* spirochetes from skin isolates revealed that BL is most often caused by *B. afzelii* and less frequently by *B. garinii*, *B. bissettii* and *B. burgdorferi sensu stricto*. (Busch *et al.*, 1996; Lenormand *et al.*, 2009; Maraspin *et al.*, 2002; Picken *et al.*, 1997; Ruzic-Sabljic *et al.*, 2000).

Acrodermatitis chronica atrophicans

Acrodermatitis chronica atrophicans (ACA) is a late skin manifestation of LB that may occur months to years after the primary infection. The lesion is characterized by a bluish-red discoloration of the skin on extremities, e.g. hands and feet (Stanek & Strle, 2003). In some patients, sclerotic lesions develop, and peripheral nerves and joints are often affected. ACA is a rare manifestation and is more often diagnosed in women than in men, and the patients are usually older than 40 years (Asbrink *et al.*, 1986; Asbrink & Hovmark, 1988). Patients with ACA usually have a positive serology and pathohistological examinations often reveal lymphocyte and plasma-cell infiltration (Asbrink *et al.*, 1986; Asbrink & Hovmark, 1988). The diagnosis can be further supported by isolation of *Borrelia* from affected skin. Characterization of *Borrelia* spirochetes from skin isolates revealed that ACA is most often caused by *B. afzelii* and less frequently by *B. garinii*, and *B. burgdorferi sensu stricto* (Busch *et al.*, 1996; Ohlenbusch *et al.*, 1996; Ruzic-Sabljic *et al.*, 2000).

Neuroborreliosis

Neuroborreliosis (NB) is a disorder of the nervous system and neurologic symptoms usually appear 4-6 weeks after the tick bite (Mygland *et al.*, 2010). NB appears in 10-15% of LB patients (Halperin, 2011) and the clinical course is highly variable and may manifest as meningitis, facial palsy, cranial neuritis and radiculitis. In a Swedish study, facial palsy, neck pain, fever and fatigue were more common in patients under the age of 40 years, whereas unspecific muscle and joint pain, radiating pain, paresthesias, vertigo, and concentration difficulties were more common in patients over the age of 40 years (Henningsson *et al.*, 2010). Laboratory confirmation of NB is hampered by the low yield of culture and of PCR examinations of cerebrospinal fluid (CSF) (Aguero-Rosenfeld *et al.*, 2005; Lebech *et al.*, 2000). The diagnosis is usually based on medical history, clinical signs and symptoms together with serological analysis of serum and CSF (Mygland *et al.*, 2010). Examination of CSF typically shows a lymphocytic pleocytosis with evidence of intrathecal production of antibodies to *Borrelia*. Characterization of *Borrelia* spirochetes from CSF revealed that NB in Europe is most often caused by *B. garinii* (69%) and less frequently by *B. burgdorferi sensu stricto* (19%), and *B. afzelii* (12%) (Wilske *et al.*, 2007). In North America, on the other hand, NB is exclusively caused by *B. burgdorferi sensu stricto*.

Lyme arthritis

Lyme arthritis (LA) is a late manifestation of LB and affects both children and adults. LA manifestations among LB patients are more common in North America (30%) than in Europe (2-7%) (Bacon *et al.*, 2007; Berglund *et al.*, 1995; Strle & Stanek, 2009). It is characterized by mono- or oligoarticular inflammation of large joints; knees, elbows, ankles, shoulders, hip. The affected joint becomes swollen and

warm and the pain is often mild or moderate (Szer *et al.*, 1991). Diagnosis of LA is based on the medical history, clinical features and serology. In ambiguous cases of LA, the diagnosis can be supported by the detection of *Borrelia* DNA in synovial tissue or synovial fluid by PCR (Stanek & Strle, 2003). Characterization of *Borrelia* spirochetes from synovial tissue and synovial fluid have revealed that *B. burgdorferi sensu stricto* is the principal but not the only *Borrelia* species involved in LA; also *B. afzelii* as well as *B. garinii* have been found in such specimens from European patients (Eiffert *et al.*, 1998; Jaulhac *et al.*, 2000; Limbach *et al.*, 2001; van der Heijden *et al.*, 1999; Vasiliu *et al.*, 1998).

Lyme carditis

Lyme carditis (LC) is a rare manifestation of LB and occurs in 1.5-10% of cases in North America and 0.5-4% in Europe (Berglund *et al.*, 1995; Strle & Stanek, 2009; Wang *et al.*, 1999b). LC appears to be three times more common among men than women (van der Linde, 1991) and patients may experience fainting, shortness of breath and/or chest pain (Steere *et al.*, 1980). LC may also be a potential cause for sudden cardiac death (CDC, 2013). Diagnosis of LC should be based on demonstration of heart involvement supported by one or more of the following; *i)* either isolation or detection of *Borrelia* spirochetes from an endomyocardial biopsy, *ii)* presence of *Borrelia* antibodies in serum or seroconversion, *iii)* presence of other LB manifestations. However, the only definitive test for diagnosing LC is by histopathology. *B. burgdorferi sensu stricto* is the only *Borrelia* species that has been detected in endomyocardial biopsies taken from LC patients both in North America and Europe (CDC, 2013; Stanek *et al.*, 1990; Strle & Stanek, 2009).

Asymptomatic *Borrelia* infection

The term asymptomatic *Borrelia* infection is used for individuals who have been exposed to *B. burgdorferi* sensu lato (i.e. detectable levels of *Borrelia* IgG antibodies in serum) but who have not recognized or do not remember having any symptoms or signs of a LB infection. In the eastern part of the county of Östergötland, Sweden, about 4% of healthy blood donors (n=408) with no history of LB had detectable levels of *Borrelia* IgG antibodies in their sera (Ekerfelt *et al.*, 2001). In Switzerland, about 26% of orienteers (n=950) had detectable levels of *Borrelia* IgG antibodies in their sera but less than 4% of them had a past history of definite or probable clinical LB (Fahrer *et al.*, 1991b). The natural history of asymptomatic *Borrelia* infection is unknown, but infection with non-invasive *Borrelia* strains as well as inter-individual differences in the immune response against *B. burgdorferi* sensu lato have been proposed as explanations (Sjowall *et al.*, 2005; Wormser *et al.*, 2001).

Post-Lyme disease syndrome

LB is usually successfully treated with antibiotics (Smith *et al.*, 2002; Wormser *et al.*, 2006) but 10-20% of LB patients with early signs of infection, e.g. EM, will still have persistent or relapsing non-specific symptoms (post-Lyme disease syndrome) 12 months after comple-tion of therapy (Barsic *et al.*, 2000; Dattwyler *et al.*, 1997; Luft *et al.*, 1996; Luger *et al.*, 1995; Nadelman *et al.*, 1992; Nowakowski *et al.*, 2003; Strle *et al.*, 1993). The most common symptoms of post-Lyme disease syndrome are fatigue, musculoskeletal pain, headache, neck stiffness, and cognitive problems. The appearance of such symptoms seems to correlate with disseminated infection, the initial severity of illness, and delayed antibiotic therapy (Asch *et al.*, 1994; Nowakowski *et al.*, 2003; Steere *et al.*, 1983). The underlying mechanisms for post-

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Lyme disease symptoms are still unknown. However, possible causes of such symptoms may include the natural evolution of response after therapy, other tick-borne infections, irreversible tissue damage, and autoimmune mechanisms. Persistent infection with *Borrelia* spirochetes has also been proposed as a cause of post-Lyme disease symptoms. However, no objective evidence of persisting *Borrelia* infection after antibiotic therapy or a benefit worthy of long-term antibiotic therapy has yet been found (Auwaerter, 2007; SBU, 2013).

Manifestation of tick-borne relapsing fever caused by *B. miyamotoi*
Human infections with *B. miyamotoi* were first described in 2011 in a report from Russia (Platonov *et al.*, 2011). In the early stage of the disease, most of the patients reported symptoms similar to those of LB, i.e. an influenza-like illness (headache, chills and muscle aches) with fever as high as 39.5°C. Only 10% of the patients exhibited EM. The time that elapsed between first noticing symptoms to hospitalization was shorter and the duration of hospitalization was twice as long (average 20 days) for patients with *B. miyamotoi*-infection, compared to patients with *B. burgdorferi* sensu lato-infection. Without antibiotic treatment, patients with *B. miyamotoi*-infection experienced relapsing febrile illness. Few cases of human infection with *B. miyamotoi* have also been reported in Europe and in North America (Gugliotta *et al.*, 2013; Hovius *et al.*, 2013; Krause *et al.*, 2013).

Strains of *B. miyamotoi* have not yet been successfully grown in culture (Barbour *et al.*, 2009). Therefore, the diagnosis currently relies on the use of PCR to detect DNA of the organism (Platonov *et al.*, 2011). However, these tests are under development and not widely available. Blood tests based on detection of antibodies also require further validation (CDC, 2014).

Treatment of Lyme borreliosis

β-Lactam or tetracycline antibiotics are normally used to treat all types of LB manifestations. β-Lactam antibiotics, e.g. penicillin and cephalosporines, are bacteriocidal, and act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. Tetracycline antibiotics, e.g. doxycycline, act by binding to the 30S subunit of bacterial ribosomes and thus inhibiting protein synthesis. The Swedish recommendations for treatment of EM patients are penicillin V, or doxycycline in case of penicillin allergy, for 10 days. For treatment of NB and LA patients, 14 days of oral doxycycline or intravenous ceftriaxone (cephalosporine) is recommended. For treatment of ACA patients, 21 days of doxycycline or penicillin V is recommended (Läkemedelsverket, 2009).

Detection of *Borrelia* spirochetes

Direct and indirect methods have been developed and used to detect *B. burgdorferi* sensu lato strains in a variety of specimen sources (infected ticks, reservoir hosts, laboratory animals, and clinical specimens). *Borrelia* spirochetes can be cultivated, visualized by microscopy and detected by PCR methods. Such direct detection techniques are extensively used in experimental and epidemiological studies, but have not been widely employed as diagnostic approaches in clinical settings. Indirect detection techniques, i.e. detection of antibodies to *Borrelia* by ELISA and immunoblot, are currently the primary tool for laboratory diagnosis of LB in humans.

Direct detection

Microscopic detection

Microscopic visualization of live *Borrelia* spirochetes offers the strongest of all proofs that an infection is present. *Borrelia burgdorferi* can be visualized directly in infected vectors, reservoir hosts, laboratory animals and clinical specimens from patients with LB using dark-field or phase-contrast microscopy. The spirochetes may also be microscopically visualized after Giemsa, Gram, immunological or silver staining of specimens (Aberer & Duray, 1991). However, the diagnostic value of direct microscopic detection of *B. burgdorferi* is limited, as the spirochete density in human clinical specimens is in general low (Aguero-Rosenfeld *et al.*, 2005). Moreover, the morphology of the *Borrelia* spirochete may be variable (Aberer & Duray, 1991), which makes it difficult to distinguish them from host tissue structures. Besides, it is nearly impossible or very difficult to determine genus or species of a spirochete by looking at the bacteria under a microscope.

Culture

Bacteria of the *Borrelia burgdorferi* sensu lato complex are slow-growing with a long generation time *in vitro*, 12-24 hours (Barbour, 1984). When they are successfully cultivated, they require a complex liquid culture medium (Barbour-Stoenner-Kelly II medium) and long incubation times (several weeks) under microaerophilic conditions. Growth is possible at lower temperatures (20°C) but is optimal at 30-37°C, where incubation at temperatures of above 39°C may reduce or prevent growth (Barbour, 1984). *Borrelia* spirochetes can be recovered from environmental specimens as well as from clinical specimens (Aguero-Rosenfeld *et al.*, 2005; Wilske, 2005). When there is an

increased risk of contamination, e.g. cultivation of spirochetes from ticks or animal tissues, antibiotics can be added to the medium. In clinical specimens, the success rate of cultivation depends on the type of specimen. The mean recovery rates of *Borrelia* from skin biopsies of patients with EM and ACA are up to 70% (Wilske *et al.*, 2007), those for blood samples and (CSF) are much lower (<10%) (Maraspin *et al.*, 2001; Ornstein *et al.*, 2001). Because of the slow procedure and the low recovery rates in body fluids, *Borrelia* cultures are rarely used as a diagnostic tool.

PCR analysis

Polymerase chain reaction (PCR) is a standard approach for direct detection of *B. burgdorferi* infection in tick vectors and in reservoir hosts. The PCR results can be qualitative (conventional PCR and/or nested PCR, real-time PCR) or quantitative (real-time PCR).

PCR targets

For qualitative PCR analysis, e.g. to determine the species of *Borrelia*, the most commonly used gene targets are *flaB* (encodes a structural protein of the internal flagella), *ospA* and *ospC* (encode the outer surface proteins A and C), and the ribosomal RNA intergenic spacers rrs(16S)-rrlA(23S) and rrf(5S)-rrl(23S) (Aguero-Rosenfeld *et al.*, 2005; Schmidt, 1997). The region of rrf(5S)-rrl(23S) is especially suitable to use for species identification. DNA sequence analysis of this region can distinguish at least eight different species within the *B. burgdorferi* sensu lato complex (Postic *et al.*, 1994). PCR amplification of the rrf(5S)-rrl(23S) yields a 225- to 266-bp long PCR product depending on the species of *B. burgdorferi* sensu lato (Masuzawa *et al.*, 1996; Postic *et al.*, 1994; Wang *et al.*, 1999a). Besides, the flanking regions of this intergenic spacer are highly uniform in the DNA se-

quence between different *Borrelia* species, which makes it suitable for primer binding. However, no amplification of this intergenic spacer has occurred with TBRF-causing *Borrelia* bacteria. To detect the TBRF species *B. miyamotoi*, rrs(16S)-rrlA(23S) can be used (Bunikis *et al.*, 2004). The major limitations of conventional PCR are poor quantification abilities and the inability to calculate the efficiency of the PCR reactions. Unlike conventional PCR, in which only the end-point PCR products are analyzed, real-time PCR provides real-time detection of PCR products by monitoring the fluorescence signal during the PCR analysis.

Real-time PCR has been used to detect and quantify *B. burgdorferi* DNA in ticks and patients (Jenkins *et al.*, 2012; O'Rourke *et al.*, 2013; Ornstein & Barbour, 2006; Piesman *et al.*, 2001; Wang, 2002). Commonly used gene targets for amplification in real-time PCR are the chromosomally encoded genes *16S* rRNA and *flaB* and also the plasmid-encoded genes of the outer surface proteins (Aguero-Rosenfeld *et al.*, 2005; Schmidt, 1997; Wang *et al.*, 1999b). The plasmids of *B. burgdorferi* are often polymorphic in sequence between species and strains, thus, making design of suitable genus-specific oligonucleotides (primers) difficult (Casjens *et al.*, 2000). Certain sequences of the *16S* rRNA gene, on the other hand, are uniform between species and strains, thus, making design of genus-specific primers easier. Furthermore, detection and quantification of 16S rRNA instead of the *16S* rRNA gene increases the detection limit (100-1000 fold) (Ornstein & Barbour, 2006). The copy number of 16S rRNA in *B. burgdorferi* cells are also less affected by the growth phase of the cell than the DNA copy numbers. Thus, rRNA copies could be used to estimate numbers of *B. burgdorferi* under different growth conditions. When the intention is to calculate spirochetal load, molecular detection of *Borrelia* 16S rRNA might be preferable to outer surface proteins, which are known to vary in degree of expression during tick feeding

(Burkot *et al.*, 1994; Fingerle *et al.*, 1995; Schwan *et al.*, 1995). However, there are limitations of using RNA instead of DNA for PCR analysis, such as the high susceptibility of RNA to degradation and nucleases during extraction and storage.

Different real-time PCR assays

The sensitivity, specificity and reproducibility of a real-time PCR assay are affected by a number of factors, including the properties of selected primers or probe, the quality of template (DNA or RNA), the amplification conditions and the detection format. Probe-based real-time PCR assays (TaqMan) have been widely used to detect and quantify *Borrelia* (Jenkins *et al.*, 2012; O'Rourke *et al.*, 2013; Ornstein & Barbour, 2006; Piesman *et al.*, 2001; Wang, 2002). Since additional hybridization of the probe is required to generate fluorescence, TaqMan assays are regarded to be more specific than SYBR Green assays in general. The real-time PCR detection format Light Upon eXtension (LUX) has never been applied to detect and quantify *Borrelia* genes. The LUX assay, compared to a SYBR green assay, offers the benefit of using a self-quenched primer with a hairpin loop structure, which would make it more specific; that is, it entails less unspecific binding and primer-dimer formation (Lowe *et al.*, 2003). Furthermore, the fluorophore is attached to the hairpin loop in the LUX setup, and thus, in contrast to the TaqMan assay, this PCR technique does not need an internal probe and is therefore a better choice if broader specificity is required. The LUX assay also has the capacity to perform melting curve analysis, which offers the possibility to discriminate between PCR products with different base pair compositions and thereby revealing false-positive samples or co-infections.

PCR as a diagnostic tool

As stated earlier, PCR technology is a standard approach for direct detection of *B. burgdorferi* infection in tick vectors and in reservoir hosts. However, PCR on clinical specimens of extracutaneous nature is, in general, of low sensitivity, with the exception of synovial fluid (a sensitivity of up to 90%) (Aguero-Rosenfeld *et al.*, 2005). Although PCR has been accepted as being of high diagnostic value for the detection of *B. burgdorferi* sensu lato in synovial fluid of patients with Lyme arthritis, its diagnostic significance for other tissues and tissue fluids has been equivocal (Aguero-Rosenfeld *et al.*, 2005; Steere, 2001). The low yield in blood (median 14% from European studies) and CSF (median 38%) could be due to the lack of spirochetemia or transient spirochetemia, very low numbers of spirochetes in blood and CSF, or the presence of PCR inhibitors in host blood and CSF (Aguero-Rosenfeld *et al.*, 2005). PCR detects DNA of both viable and non-viable *Borrelia* bacteria, making it impossible to distinguish if an infection is active or not. PCR is not a routine method in diagnosing LB but can be valuable as a supplementary diagnostic tool (Aguero-Rosenfeld *et al.*, 2005).

Indirect detection

Immunofluorescent antibody assay

Immunofluorescent antibody assay (IFA) is a method that was earlier used to detect *Borrelia*-specific antibodies in serum of LB patients. Cultured *Borrelia* spirochetes are fixed onto glass slides and incubated with patient serum. Possible *Borrelia*-specific antibodies in the serum bind to the fixed *Borrelia* spirochetes. After the addition of Fluorescein isothiocyanate (FITC) labeled secondary antibodies, the presence of antibodies bound to spirochetes can be detected by fluo-

rescence microscopy (Aguero-Rosenfeld *et al.*, 2005). Besides that IFA is labor intensive, it may also be difficult to objectively read and interpret the results. IFA may still be in use in experimental studies, but as a diagnostic tool it has been replaced by ELISA and immunblot methods, described below (Aguero-Rosenfeld *et al.*, 2005).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is the most commonly used method to detect antibodies of *B. burgdorferi* sensu lato. It is inexpensive, automated and easy to perform. Diluted patient fluid (serum or CSF) is incubated in microwells that are pre-coated with a borrelial antigen (sonicated antigens, purified native antigens, recombinant antigens or synthetic peptides). Antigen-specific antibodies bind to the antigen and secondary enzyme-linked antibodies are added which bind to the complex. When substrate for the enzyme is added, a visible fluorescent signal is produced which can be measured and associated with the concentration of patient antibodies to the investigated antigen. The degree of fluorescence is read by an automated system, which allows an objective determination of antibodies (Aguero-Rosenfeld *et al.*, 2005). High titers of either IgG or IgM antibodies to borrelial antigens indicate an ongoing disease, but IgM antibodies may remain after the initial infection, and IgG antibodies may remain for years (Burdash & Fernandes, 1991). One shortcoming of ELISA is that it may cross-react with antigens from other bacteria (e.g other spirochetes such as *Treponema* and *Leptospira*) than *B. burgdorferi* sensu lato (Magnarelli *et al.*, 2012). Another important limitation of ELISA is the lack of standardization: commercially available ELISA tests use different antigenic composition and detect different immunoglobulin classes (Aguero-Rosenfeld *et al.*, 2005; Ekerfelt *et al.*, 2004).

Western blot

Western blot (WB) is based on the use of antigens separated by molecular size and detection of antibody reactivity to these specific antigens. Borrelial antigenic proteins are transferred to a nitrocellulose membrane and incubated with patient serum. Antigen-specific antibodies present in the serum bind to their respective antigens. The bound antibodies may be visualized in a way similar to ELISA. WB is usually applied as a confirmatory test to equivocal or positive ELISA test. However, WB is limited by the lack of standardization of the antigen source, visual scoring and subjective interpretation of band intensity (Aguero-Rosenfeld *et al.*, 2005). Criteria for WB interpretation have been established in North America (CDC, 1995), but WB interpretation is more complicated in Europe, due to diversity of *Borrelia* species, and consensus on criteria has not been reached (Hauser *et al.*, 1997; Robertson *et al.*, 2000).

Enzyme-linked immunospot

Enzyme-linked immunospot (ELISPOT) can be used for visualization of *Borrelia*-specific cytokine secretion by reactive lymphocytes (Czerkinsky *et al.*, 1988; Ekerfelt *et al.*, 1997; Forsberg *et al.*, 1995). Microplates are coated with monoclonal anti-human antibodies and incubated together with lymphocytes and borrelial antigens. Cytokines secreted by activated lymphocytes are bound by the coated antibodies. This can further be visualized as a spot by a secondary antibody. Each spot represents a single reactive cell and therefore the ELISPOT assay provides both qualitative (type of immune protein) and quantitative (number of responding cells) information. This method is currently used exclusively for research purposes and not as a routine diagnostic test.

Epidemiology

Today, LB is the most common diagnosed tick-borne disease in the temperate regions of the Northern hemisphere. Around 86,000 people are estimated to get an LB diagnose every year (Hubalek, 2009). Approximately, 77% of all LB cases are diagnosed in Europe, 19% in North America, 4% in Asia, and < 1% in parts of North Africa. However, these figures are highly uncertain, since numerous of countries (including Sweden) lack a mandatory LB notification system. The age distribution of the LB disease in most countries is usually bimodal with the first (lower) maximum occurring in children 5-9 years old, and the second (higher) maximum in persons aged 50-64 years (Hubalek, 2009).

In an epidemiological investigation of LB conducted in southern Sweden 1995, Berglund and co-workers reported an annual incidence of 69 LB cases per 100,000 inhabitants (Berglund *et al.*, 1995). Inhabitants in endemic areas of this region may have a *B. burgdorferi* IgG seroprevalence between 19% and 21% (Berglund & Eitrem, 1993; Berglund *et al.*, 1996). The Åland Islands, on the other hand, is a hyper-endemic region with an annual LB incidence of about 1000/100,000 inhabitants in years 2000-2012 (THI, 2013), and the inhabitants may have a *B. burgdorferi* IgG seroprevalence of 20% (Carlsson *et al.*, 1998).

The incidence of LB in humans is dependent on many factors such as the distribution and density of tick population, the prevalence of pathogenic *Borrelia* species in the ticks, as well as the extent of human activity in tick-infested areas (Hubalek, 2009; Mejlon & Jaenson, 1993). Information about the clinical and serological outcome, e.g. incidence of LB, and prevalence of *Borrelia*-antibodies after a tick bite

would therefore be valuable when possible risk factors of developing *Borrelia*-infections are investigated.

Prevention of tick bites and Lyme borreliosis

The most efficient way to reduce the risk of tick-borne infections is to avoid tick bites. Such strategies include avoidance of tick-infested areas, use of protective clothing and use of tick repellents on either skin or clothing, and routine bodily checks for ticks (Clark & Hu, 2008). Tick repellents containing DEET (diethyl-3-methylbenzamide) may repel >85% of tick attacks one hour after application but its effectiveness may decrease over longer time periods (Pretorius *et al.*, 2003).

If a tick is found attached to the skin it is advisable to remove it as soon as possible to reduce the risk of a potential *Borrelia* spirochete transmission. A person who has removed an attached tick from the skin should observe the area of the bite for expanding redness, which would suggest EM, the characteristic rash of LB. Since many tick bites may go unnoticed it is a good advice to learn the signs and symptoms of LB, especially for people living in highly tick-infested areas. Another approach to reduce the risk for tick bites is to control the tick population. Controlling ticks by habitat manipulation and insecticides may be possible in smaller areas (gardens, public parks, small islands) but may be more difficult in larger areas (Clark & Hu, 2008).

At present time there is no prophylactic vaccine available against LB. In 1998, however, a vaccine called LYMErix, produced by the British pharmaceutical company SmithKline Beecham (today: GlaxoSmithKline), became commercially available in USA but was voluntarily withdrawn from the market in 2002. The company cited poor

market performance (Nigrovic & Thompson, 2007), but the real reason was rather fear of lawsuits: reports of severe adverse reactions occurring after vaccination started to appear (Lathrop *et al.*, 2002). The demise of LYMErix has not ended research on new Lyme vaccine candidates. The American health care company Baxter is currently working on a new candidate (Wressnigg *et al.*, 2013). This vaccine is "designed" to induce killing of *Borrelia* spirochetes while they are still inside the feeding tick. This is supposed to prevent a transmission of spirochetes from the tick to the tick-bitten person. A similar vaccine was used to vaccinate wild white-footed mice in a field-study conducted in North America. This significantly reduced the prevalence of *B. burgdorferi sensu stricto* in nymphal ticks (*I. scapularis*) collected at the sites the following year (Tsao *et al.*, 2004).

Aims

In the background we learned that the tick-population in Sweden appears to increase and wherever ticks and humans exist together there is always a risk for tick bites and tick-borne infections such as LB. In Sweden, there are gaps in the current knowledge regarding ticks, *Borrelia* bacteria and the clinical situation of LB. For instance, there is no data regarding the prevalence of *Borrelia* species among ticks that bite humans. Such information is crucial to fully understand the epidemiology of LB. In Sweden, there is no mandatory LB notification system and the actual clinical situation of LB is unknown. In addition, an LB infection starts with a tick bite and our understanding of the ecological interactions between ticks and humans is both fragmented and incomplete. If we can improve our understanding of these issues, we may be able to provide information that may reduce the number of tick bites as well as the number of LB cases.

The overall aims of the thesis are to increase our understanding of the epidemiology of the *Borrelia* bacteria, and the ecology of ticks, as well as to increase our understanding of the clinical situation of LB. Furthermore, to provide information that can help reduce the number of tick bites and the number of LB cases in tick-infested areas. In order to achieve these goals, three parts (**1-3**) of the interactions between humans, ticks, and *Borrelia* bacteria will be thoroughly investigated (Figure 6):

Part 1: The interactions between humans and ticks (paper I)
Are there temporal differences in exposure to tick bites in different parts of Sweden and the Åland Islands? Which life stages of the tick are dominating regarding tick bites on humans and are there temporal differences in exposure to the different life stages? Where on

the body do tick-bitten persons find attached ticks? How long time does it take for a person to detect an attached tick?

Part 2: The interactions between ticks and *Borrelia* bacteria (paper II and III)

What is the prevalence of Borrelia-*infected ticks that bite humans? Which* Borrelia *species are present in ticks that bite humans? How is the* Borrelia *load (the number of* Borrelia *cells) in a tick that feeds on a human affected by the duration time of tick feeding?*

Part 3: The interactions between *Borrelia* bacteria and humans (paper IV)

What are the clinical outcome (prognosis) and the serological response after a bite by a Borrelia-*infected tick? Are there gender- and geographical differences in the* Borrelia *seroprevalence among tick-bitten persons? Which factors may facilitate* Borrelia *transmission from tick to human and give rise to LB?*

To examine these parts, the Tick-Borne Diseases (TBD) STING-study was set up and carried out.
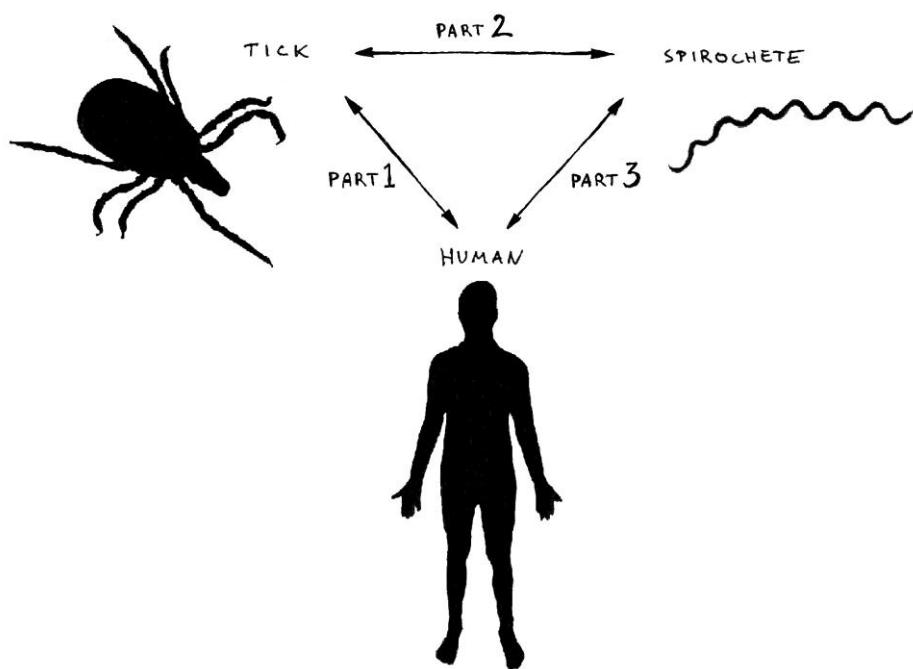
Figure 6. Schematic outline of the studied interactions.

The Tick-Borne Diseases STING-study

This thesis is based on four papers (**I-IV**), and each one of them is related to the Tick-Borne Diseases (TBD) STING-study. In the following section, the general design of the TBD STING-study is described. Full details regarding material and methods used in this thesis can be found in each paper.

Design

The TBD STING-study has followed a multidisciplinary approach to understand the diverse, but specific, interactions between ticks, humans and tick-borne pathogens. The goal is to determine the prevalence of potentially pathogenic bacteria and viruses in ticks that have bitten humans, such as *Borrelia* spp, Tick-borne encephalitis virus, *Anaplasma* spp. *Rickettsia* spp. and *Candidatus* Neoehrlichia mikurensis, and to evaluate if life stage of the tick, its pathogen content, its attachment site on the human, and its duration of feeding influence the risk of pathogen transmission and the development of an infection. To investigate these tasks, ticks that have bitten humans, questionnaires, and blood samples taken from tick-bitten persons are collected from primary health care centers (PHCs) since June 2007. At the time of this thesis, about 5,000 tick-bitten persons and "their" ticks have been included in the study, and more than 60 PHCs located in Sweden (the counties of Småland, Blekinge, Skåne, Västra Götaland, Östergötland, Dalarna, Dalsland and Västerbotten), the Åland Islands, Finland (all archipelago municipalities) and in Norway (county of Aust-Agder) have been involved.

Material collection

Recently tick-bitten persons, aged 18 years or older, are recruited to the TBD STING-study by advertisements and articles on local television, on radio, in newspapers, and on internet (see www.stingstudien.se). Recruitment posters are also displayed in public places, libraries, grocery stores, PHCs, pharmacies, and train- and bus stations. The tick-bitten persons are asked to bring "their" tick to a PHC that is recruited for the TBD STING-study. Before a person is asked to participate, a member of the staff from the PHC inform about the general outline and the aims of the TBD STING-study. The tick-bitten person signs a written consent to participate, provides personal data (such as age and gender), donates the removed tick, provides a blood sample, and completes a questionnaire. The participant receives a plastic tube to collect any ticks that may possibly bite the participant again during the three-month study period. It is advised to store the plastic tube in a fridge during the whole study period. The participant also receives a small card which is used if the participant visits a health care provider during the study period; he/she is advised to give this card to the attending physician. The card contains instructions for the physician to take blood samples from the TBD STING-study participant and send these to the laboratory at the Division of Clinical Immunology, Linköping University, Sweden.

Three months after study inclusion, the participant receives a letter where he/she is asked to revisit the PHC. At the PHC, the 10 ml plastic tube (containing ticks that may have bitten the participant during the study period) is handed in, a new blood sample is taken and a second questionnaire is filled in.

All collected material and questionnaires at the PHC are sent to the laboratories at the Division of Medical Microbiology and the Division

of Clinical Immunology, Linköping University, within three days. Upon arrival, blood samples and ticks are frozen (-70°C) until analysis.

Participants of the TBD STING-study are allowed to discontinue their participation at any time, when so requested, all samples from that person are discarded.

Analysis of ticks, tick-borne pathogens, blood sera, questionnaires and medical records

A schematic outline of the analysis of ticks, tick-borne pathogens, blood sera, questionnaires and medical records is shown in Figure 7.

The ticks

Each individual tick is thawed and photographed dorsally and ventrally, to determine species, life stage, and sex of adults (**I**). To estimate the duration of feeding for adult female ticks and nymphs of *I. ricinus*, the scutal index (the ratio of the length of the idiosoma to the width of the scutum) or the coxal index (the ratio of the distance between the basal coxae of the fourth pair of legs to the width of the scutum) are calculated (**I**). Immediately after the tick has been measured, total nucleic acids from the tick are extracted, purified and isolated using automatically physical and chemical methods. This is followed by cDNA synthesis (**III**).

The tick-borne pathogens

The cDNA is used as template in different real-time PCR assays, developed to detect and quantify tick-borne pathogens. Detection and quantification of the *Borrelia* bacteria is performed using a real-time PCR assay based on the LUX system (**II**). Primers are designed to target and amplify a highly conserved region of the *16S* rRNA gene of *Borrelia* spp. Species determination of the *Borrelia* bacteria is performed using conventional nested PCR assays (**II**). Primers are designed to target and amplify the borrelial intergenic spacers of rrf(5S)-rrl(23S) and rrs(16S)-rrlA(23S). Amplified PCR products are sequenced, followed by sequence analysis.

In order to assure the reproducibility of the total nucleic acid extraction and cDNA synthesis and to prevent false-negative results, randomly selected tick samples in each extraction batch are analyzed for the presence of *Ixodes* tick mitochondrial *16S* rRNA gene with a SYBR-green real-time PCR assay (**III**).

The blood sera

Sera taken from the participants, both at the time of inclusion and at the three-month follow-up visit, are screened for the presence of antibodies against different tick-borne pathogens using ELISA tests in order to determine possible seroconversion. For analysis of *Borrelia* antibodies, two different ELISA tests are used (**IV**): 1) IDEIA™ *B. burgdorferi* IgG (Oxoid) detects IgG anti-flagellum antibodies, 2) Immunetics® C6 *B. burgdorferi* IgM/IgG (Lyme) detects IgM/IgG anti-C6 antibodies. A seroconversion, in either one or both of the ELISA tests, is verified by analyzing the sera with an immunoblot test;

recomLine *Borrelia* IgG (**IV**). A verified seroconversion is defined as a current *Borrelia* infection.

The questionnaires and medical records

The first questionnaire (see Additional file 1, **I**), answered by the participants at enrollment, includes questions about the date of detection of the attached tick, assumed duration of the tick attachment, the geographical location of the tick bite, the anatomical location of the tick bite, estimated numbers of tick bites contracted earlier that season, previous tick-borne infections, the general health status among other questions. The second questionnaire (see Additional file 2, **I**), answered by the participant three months after enrollment, includes questions about new tick bites during the three-month study period, the general health during the three-month study period, symptoms possibly associated with LB (headache, fatigue, fever, neck pain, loss of appetite, nausea, weight loss, vertigo, concentration difficulties, radiating pain, myalgia/arthralgia, and numbness), and whether or not, the participant has visited a health care provider because of such symptoms. In such cases, the medical record of the participant is obtained and scrutinized in detail by physicians with long-time clinical experience of LB and other tick-borne infections.
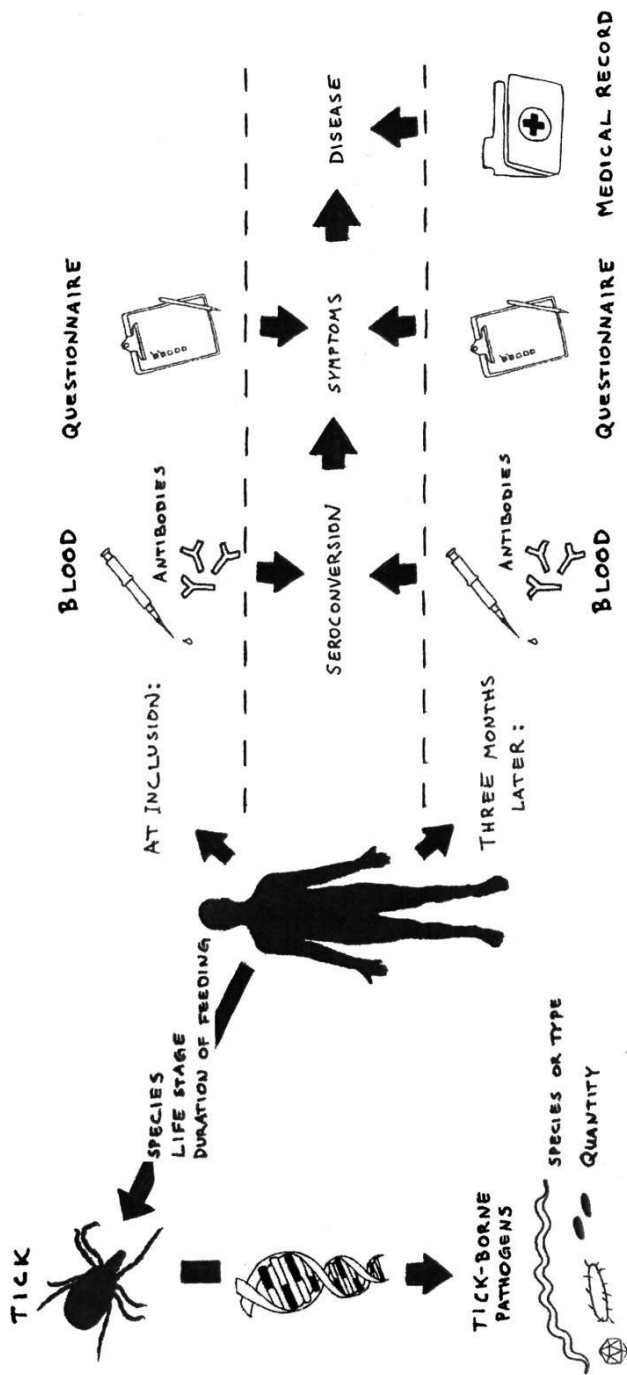
Figure 7. Schematic outline of the analysis of ticks, tick-borne pathogens, blood, questionnaires, and medical records.

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Statistics

GraphPad Prism for Windows, version 5.00 was used for the statistical analyses in this thesis (**I-IV**). Data was summarized as percentage for categorical variables and as medians with interquartile range (IQR) for numerical variables. The categorical variables were analyzed by using the Chi-square test or the Fisher's exact test. The numerical variables were analyzed by using the non-parametric Mann-Whitney test (comparison of two groups) or the non-parametric Kruskal-Wallis test (comparisons of three or more groups). The Dunn's test was applied for post-hoc comparisons after a significant Kruskal-Wallis test. Correlations between parameters were calculated using the Spearman rank correlation test. P-values ≤ 0.05 were considered statistically significant.

Ethics

Ethical permission for the TBD STING-study was approved 2006 by Regional Ethical Review Board in Linköping (M132-06), and by the local Ethical Committee of Åland, Finland, 2008-05-23.

Part 1. The interactions between humans and ticks – results and discussion (paper I)

In an effort to investigate which species and life stages of ticks that bite people, when people become infested by ticks, where on the human body that ticks generally bite, and how long time that elapse before an attached tick is detected and removed, we collected and analysed ticks (n = 2110) that had been found attached to the skin of persons (n = 1770) that participated in the TBD STING-study between 2008 and 2009. This was related to the participants' answers to the questionnaires. This included participants from Southernmost Sweden (n = 477), South Central Sweden (n = 688), Northern Sweden (n = 15), and from the Åland Islands (n = 590) (Fig. 1, **I**).

Are there temporal differences in exposure to tick bites in different parts of Sweden and the Åland Islands?

Ixodes ricinus ticks become active when the temperature is above 4°C (Duffy & Campbell, 1994), and the risk of contracting tick bites is higher during times of the year when ticks are host-seeking. All the collected ticks in the TBD STING-study belonged to the species *I. ricinus*. Most participants of the TBD STING-study were exposed to tick bites from April to November, with peaks in early and late summer (Fig. 2, **I**). However, geographical differences were observed. In Southernmost Sweden, South Central Sweden and on the Åland Islands, participants were exposed to tick bites approximately during the same time period of the years (April/May to October/November). In Northern Sweden, on the other hand, participants recorded tick

bites during a shorter time period (June/July to August/September). This, along with the low number of tick bites (n = 15) recorded by the participants in Northern Sweden, indicate an overall low tick burden in this area. However, a warmer climate with milder winters and a prolonged vegetation period have permitted important *I. ricinus* maintenance hosts, particularly roe deer (*Capreolus capreolus*), to spread to and inhabit previously climatically suboptimal areas in the northern parts of Sweden. This may, at least partly, explain why the range and abundance of *I. ricinus* in northern Sweden has increased during the last 30 years (Jaenson & Lindgren, 2011). Consequently, this has led to an increased risk for tick bites among the inhabitants of northern Sweden.

The participants of the TBD STING-study were, in general, exposed to tick bites from April to November, but this does not mean that ticks are not active the other months of the year. The seasonal tick infestation pattern on the TBD STING-study participants, is influenced not only by varying activities of people, e.g. when people tend to visit tick-infested areas for berry- or mushroom picking or other purposes but also by the seasonal host-seeking activity pattern of the tick. The host-seeking pattern is variable and not yet fully understood. It is influenced by several biotic and abiotic factors including vegetation type, density and variety of hosts, weather and climate, and the life stage of the tick.

To reduce the number of tick bites and tick-borne infections among humans in tick-infested areas, one may reduce the number of important tick maintenance hosts, such as roe deer (*Capreolus capreolus*). Several studies report strong correlations between roe deer density and tick density (Daniels *et al.*, 1993; Jensen *et al.*, 2000; Wilson *et al.*, 1984). Reasonably, a lower density of roe deer, results in a lower density of ticks and therefore a reduced risk for tick bites

and tick-borne infections among humans. Another approach to lower the density of ticks is to control them by habitat manipulation or using acaricides. However, to do this in a larger area must be extremely difficult and it is probably easier carried out in a smaller areas (Clark & Hu, 2008).

Which life stages of the tick are dominating regarding tick bites on humans and are there temporal differences in exposure to the different life stages?

Most of the tick bites (>70%) recorded by the TBD STING-study participants were caused by *I. ricinus* ticks in the nymphal stage (Table 1, **I**). In general, the seasonal nymph infestation pattern on the participants during 2008 and 2009 was bimodal with peaks in June-July and August (Fig. 3, **I**). The reduction of nymph infestation between the peaks is probably caused by a lower host-seeking activity among the nymphs, which are particular vulnerable to desiccation, and do not tend to seek hosts during the hottest and driest part of the summer. This was reported in two different field-studies on host-seeking *I. ricinus* nymphs in south-central Sweden (Mejlon & Jaenson, 1993; Talleklint & Jaenson, 1996).

About one fourth of the tick bites (24%) recorded by the TBD STING-study participants were caused by *I. ricinus* ticks in the adult stage (Table 1, **I**). The seasonal adult tick infestation pattern on the participants was unimodal rather than bimodal and peaked in July-August (Fig. 3, **I**). The seasonal host-seeking pattern for adult ticks of *I. ricinus* has also been found to be unimodal rather than bimodal (Mejlon & Jaenson, 1993). The reason for this could be that adult ticks, compared to the smaller nymphs, may have a greater resistance to desic-

cation and therefore able to seek hosts even during the hottest and driest part of the summer.

Only 4% of the tick bites recorded by the TBD STING-study participants were caused by *I. ricinus* ticks in the larval stage (Table 1, **I**). Due to the low number of larvae, no conclusions about their seasonal infestation pattern on the participants could be drawn. However, the host-seeking pattern for *I. ricinus* larvae has been found to be bimodal, like the pattern for host-seeking nymphs (Mejlon & Jaenson, 1993). One explanation for the low number of larvae recorded by the participants can be that larvae usually "prefer" to feed on smaller mammals such as rodents and shrews (Talleklint & Jaenson, 1997) and on small ground-frequenting birds (Olsen *et al.*, 1995). Another explanation could be that the larva stage of the tick is inconspicuous and may be easily overlooked when it is attached to the skin of a person. Interestingly, the participants of the TBD STING-study who were infested by larvae recorded, in general, more than one larva on their skin (unpublished results). The reason for this could be that the tick larvae usually occur in aggregations in small spots in nature (Daniels *et al.*, 1989). This means that a person bitten by a larva has come across such highly larvae-infested spot and probably encountered many larvae at the same time.

Since adult ticks are more often infected with the *Borrelia* bacteria than nymphs, and nymphs are more often infected than larvae (**II, III**, further discussed in **Part II: The interactions between ticks and *Borrelia* bacteria**), one may assume that there is a similar descending order in their importance of *Borrelia* bacteria transmission to humans. However, most of the tick bites recorded by the TBD STING-study participants were caused by nymphs (>70%) and not by adult ticks (24%). This indicates that nymphs rather than adult ticks are the most important source of *Borrelia* infection for humans. On the other

hand, we do not know if nymphs and adult ticks have the same ability to transmit the *Borrelia* bacteria to human hosts when they feed. Nevertheless, the TBD STING-study participants only removed ticks that they detected. In reality, they may have had a different proportion of the three life stages attached to their bodies. One should bear in mind that one third up to two thirds of tick bites on humans may go unnoticed (Strle et al., 1996; Strle et al., 2002).

Where on the body do tick-bitten persons find attached ticks?

People may be bitten by ticks on any site of the human body. Most of the tick bites recorded by the TBD STING-study participants took place on their legs (50%, Fig. 5, **I**). A similar observation was noticed for humans bitten by *I. ricinus* ticks in Switzerland (Hugli *et al.*, 2009) as well as for humans bitten by *I. scapularis* ticks in the United States (Falco *et al.*, 1996). The height of the legs is approximately within the same height above the ground (< 100 cm) where *I. ricinus* ticks usually quest in the vegetation (Mejlon & Jaenson, 1997). This suggests that most ticks, searching for an optimal attachment site on a human host, will walk only a short vertical distance before they will penetrate the skin and start to feed. The second most common anatomical site of tick attachment recorded by the TBD STING-study participants was torso/dorsum (22%), followed by arms (18%), groin or genital (6%), and head or neck (4%). These proportions correspond arbitrarily to the anatomical distribution of EM that was found among LB patients (n = 118): legs (64%), torso/dorsum (25%), arms (10%) and genitalia (1.7%) (Bennet *et al.*, 2006).

Among the participants of the TBD STING-study, a significantly higher proportion of men (9%) than women (5%) recorded ticks attached to the groin/genital area (Fig. 5, **I**). Berglund and co-workers also found

that men were more commonly bitten in the genital region compared to women (Berglund *et al.*, 1995). Women, on the other hand, recorded a significantly higher proportion of tick bites in the head and neck area (5%) compared to men (1%). This may reflect behavioral and physiological differences between the genders and may be apparent when coming in contact with the tick and/or examining the body to detect attached ticks or in the capacity to sense weak mechanical stimuli that touch the skin, e.g. from a tick walking on the skin. In general, the density of hairs (the pilosity) on the body of men is greater, and their hairs are often thicker and longer compared to those on women. One evolutionary advantage of having an intense pilosity is, most likely, that the hairs will effectively sense potentially harmful ectoparasites (ticks, mites, lice, fleas, etc). Thus, in view of the more intense pilosity on men, compared to women, men are likely to sense, detect and to remove any tick, that has just begun to search for a feeding site on his skin, sooner than another tick that has simultaneously begun to search for a feeding site on the skin of a woman. However, once a tick finally has penetrated the skin it appears that women rather than men detect and remove the tick sooner (further discussed below). Nevertheless, *I. ricinus* ticks usually quest on the ground or on low vegetation (< 100 cm) (Mejlon & Jaenson, 1997), so they will usually start their search for a feeding site on a human host from below the waistline, usually on the legs. The more intense pilosity on men may explain why fewer ticks manage to reach the head and neck area of men, compared to the same area of women.

A tick bite may lead to a tick-borne infection such as LB. A deeper knowledge about where on the human body ticks generally are detected can be used to inform people of where on the body one should check for ticks. Information regarding where on the body ticks usually are detected may also be used to increase the effectiveness

of other prophylactic actions to reduce the risk for tick bites. This may include development of protective clothing and guidance of where on the human body or where on the clothes tick repellents should be used.

How long time does it take for a person to detect an attached tick?

Ixodes ticks may feed on a host for long periods (2–15 days) and the duration of tick feeding is closely associated with the efficacy of *Borrelia* bacteria transmission from tick to host (Crippa *et al.*, 2002; Kahl *et al.*, 1998). Piesman and co-workers observed that *Borrelia*-infected *I. scapularis* nymphs transmitted the infection to 1 of 14 rodents exposed for 24 hours, 5 of 14 rodents exposed for 48 hours, and 13 of 14 rodents exposed for greater than or equal to 72 hours (Piesman *et al.*, 1987). Among the TBD STING-study participants, 63% detected and removed "their" ticks later than 24 hours of tick feeding (**I**), i.e. after a more extended tick feeding period which potentially would be more permissive for a *Borrelia* transmission. It is important to note, however, that only few of the participants became infected with *B. burgdorferi* sensu lato after a bite by a *Borrelia*-infected tick (**IV**, further discussed in **Part III: The interactions between *Borrelia* bacteria and humans**). One explanation for the low number of *Borrelia* infections among the participants could be that few ticks (5%) were still attached to the skin after 72 hours (Table 3, **I**).

The location of tick attachment site on the body influenced how soon a tick was detected. Ticks attached on the head/neck area, or in the groin/genital area were detectable with more difficulty than ticks attached to other areas of the body (Table 2, **I**). This has previously been shown for *I. ricinus* nymphs as well as for *I. scapularis* adult fe-

male ticks attached to the head/neck area of humans (Falco *et al.*, 1996; Hugli *et al.*, 2009).

In general, the women in the TBD STING-study detected "their" ticks earlier than the men (Table 3, **I**): More than 40% of the women and around 30% of the men removed the ticks within 24 hours of tick attachment. The reason why men detect "their" ticks later than women is unknown but may be due to behavioral, physiological, and/or immunological differences between the genders. Nevertheless, it suggests a higher risk of *Borrelia*-transmission to men if they are bitten by *Borrelia*-infected ticks which in turn may, at least partly, explain why the men of the TBD STING-study had a higher seroprevalence of *B. burgdorferi* sensu lato IgG antibodies in their sera compared to the women (**IV**, further discussed in **Part III: The interactions between *Borrelia* bacteria and humans**).

In general, older participants in the TBD STING-study detected "their" nymphs later than the younger ones (Table 4, **I**): The proportion of nymphs attached > 24 hours increased with increasing age; participants aged 30-39 years accounted for the smallest percentage (46%) of nymphs attached > 24 hours, while participants >80 years accounted for the largest percentage (75%) of nymphs attached > 24 hours. Similar observations were reported in a study on *I. scapularis* nymphs removed from humans in United States (Falco et al., 1996). Attachment times of nymphs increased significantly with the age of the victim and about 50% of all tick-bitten participants >50 years detected their nymphs after more than 48 hours of attachment. The reason why older tick-bitten persons detect "their" ticks later than younger ones could be that older people are likely to have poorer eye vision and impaired physical sensitivity. A longer duration of tick attachment on "older" persons may, at least partly, explain why "older"

persons (aged 50-64 years) constitute a large risk group of LB in most countries (Hubalek, 2009).

Surprisingly, the same proportions of adult female ticks and nymphs were detected and removed later than 24 hours. We had expected that the larger, adult female ticks would be detected sooner than the smaller and more inconspicuous nymphs. This was reported in a study on *I. ricinus* ticks feeding on humans in Switzerland (Hugli et al., 2009) as well as in a study on *I. scapularis* ticks feeding on humans in the United States (Falco et al., 1996). Both studies found that adult female ticks were detected and removed sooner than nymphs. In these studies, however, only scutal index was applied to relate changes in size and dimension of the tick to its duration of feeding. In the TBD STING-study, we applied both the scutal- and the coxal indices to estimate the duration of tick feeding (**I**). The reason for this was that the coxal index, compared to the scutal index, has a greater sensitivity during the early part of the feeding period, i.e. it detects a faster change in tick size and dimension when the tick feeds (Gray et al., 2005). Thus an adult female tick that appears to have a short feeding period as estimated with the scutal index may actually have a longer feeding period when the duration of feeding is estimated with the coxal index. Therefore, the use of the coxal index may explain why we found a higher proportion of adult female ticks attached for more than 24 hours than the other studies did. The discrepancies between the TBD STING-study (**I**) and the other studies could also be due to differences in the general knowledge of the participants and their awareness about tick-infested habitats.

To the naked eye the larvae look like specks of soot, while nymphs look like poppy seeds. Adult ticks, with their eight legs, look like small spiders. Despite their different morphological characteristics, adult female ticks and nymphs of *I. ricinus* attached to the skin of the par-

ticipants of the TBD STING-study seemed to be detected and re-moved equally fast. The time it took for the participants to detect and remove a skin-attached tick also seemed to be influenced by the age and gender of the tick-bitten person as well as the location of tick attachment site on the body. An early finding and a prompt removal of any attached tick is a prudent public health measure to reduce the risk of *Borrelia* transmission and the risk of developing LB. Our results indicate that some people (especially men and "older" people) are not inspecting themselves adequately for the presence of attached ticks. Information regarding this issue, targeted to these "vulnerable" groups may reduce their risk of developing LB.

Part 2. The interactions between ticks and *Borrelia* bacteria – results and discussion (paper II and III)

In an effort to investigate the *Borrelia* species and the *Borrelia* load (number of *Borrelia* cells) in ticks that have bitten humans, we collected and analyzed ticks (n = 2154) that had been found attached to the skin of persons that participated in the TBD STING-study 2008 and 2009. The *Borrelia* species of the *Borrelia*-infected ticks were determined and *Borrelia* cells were quantified. Analyzed ticks were collected from Southernmost Sweden, South Central Sweden, Northern Sweden, and the Åland Islands (Fig. 1 and Table 1, **III**).

What is the prevalence of Borrelia-*infected ticks that bite humans?*

Overall, 26% of all tick bites (556 of 2154) on the TBD STING-study participants between 2008 and 2009 were caused by *Borrelia*-infected ticks (Table 1, **III**). The prevalence varied between the different life stages of the ticks and between the studied geographical areas.

None of the larvae (n = 87) was *Borrelia*-infected. This is in agreement with previous results and indicates that transovarial transmission of *B. burgdorferi* sensu lato in *I. ricinus* rarely, if ever, occurs (Richter *et al.*, 2012; Rollend *et al.*, 2013). In contrast, 25% of the nymphs were *Borrelia*-infected and the *Borrelia* prevalence slightly varied between the studied areas (22-26%, Table 1, **III**). Adult ticks, on the other hand, were more often infected (35%), and the *Borrelia* prevalence varied considerably between the studied areas (0-40%). The higher *Borrelia* prevalence found in the adult ticks compared to

the nymphs is in agreement with previous reports and is probably related to the higher number of blood meals ingested by the adult ticks (Rauter & Hartung, 2005).

A *Borrelia*-infected nymph acquires the infection most likely from its previous blood meal, either by feeding on a systemically infected host (e.g. bank voles or mice) or by co-feeding with infected nymphs or with infected adult female ticks. Since the majority of all tick bites on the TBD STING-study participants were caused by nymphs (>70%, **I**) and that every fourth nymph was infected (**III**), it is relevant to identify the *Borrelia* reservoir hosts that are responsible for transmitting the infection to the nymphs. This may be achieved by analyzing the previous blood-meal of *Borrelia*-infected ticks, in which host DNA can be identified (Moran Cadenas *et al.*, 2007). Identification of important *Borrelia* reservoir hosts is crucial for disease-control measures, e.g. when to reduce the number of such hosts or when to vaccinate them. Vaccinated *Borrelia* reservoir hosts may significantly reduce the prevalence of *Borrelia* bacteria in ticks (Tsao *et al.*, 2004). This would lead to lower circulation of *Borrelia* bacteria in nature and consequently to a reduced risk for humans contracting *Borrelia* infections after tick bites (Voordouw *et al.*, 2013). Identification of *Borrelia* reservoir hosts may also provide a deeper understanding of the *Borrelia* circulation within the natural cycle between ticks and hosts.

Which Borrelia *species are present in ticks that bite humans?*

In total, six species of the *B. burgdorferi* sensu lato complex and one TBRF species were detected in the ticks collected from tick-bitten persons (Table 1, **II** and Table 1, **III**). *B. afzelii*, which is the cause of most cases of EM, ACA, and BL in Europe, was the predominant species and was detected in 50% of all ticks that contained the *Borrelia*

bacteria. The second most common species was *B. garinii* (19%), which is the cause of most cases of NB in Europe. The other detected *Borrelia* species were *B. valaisiana* (7%), *B. burgdorferi sensu stricto* (4%), *B. miyamotoi* (2%), *B. spielmanii* (1%) and *B. lusitaniae* (1%). *B. spielmanii* and *B. lusitaniae* were detected for the first time in the studied areas. A similar descending order in distribution of *Borrelia* species was observed in most of the studied geographical areas (Table 1, **III**). In addition, 2% of the ticks had a mixed infection of *Borrelia* species. Interestingly, 15% of the ticks were infected with *Borrelia* that could not be determined to species. These ticks were, in general, infected with lower number of *Borrelia* cells, compared to ticks infected with typeable *Borrelia* species (**III**). This may, at least partly, explain why PCR products, used to determine *Borrelia* species, were not amplified in the conventional PCR assays.

All *Borrelia* species, detected in ticks collected from the tick-bitten TBD STING-study participants, should be considered as potentially human pathogenic; they have all previously been detected in clinical samples taken from *Borrelia*-infected patients. Knowledge about the *Borrelia* species that circulates among ticks that bite humans should be valuable when diagnostic tools are developed. We detected the TBRF-causing species *B. miyamotoi* in such ticks; most of them (8 out of 11) were detected in ticks collected from South Central Sweden (Table 1, **III**). Many of the diagnostic methods (both serological and molecular) are not developed to detect the presence of this species. At least one TBRF case in Europe has been reported, i.e. in the Netherlands (Hovius *et al.*, 2013), and Swedish clinicians should, therefore, be advised to be observant for the signs and symptoms of TBRF among patients, since this species is present in ticks that bite humans in Sweden.

Studying *Borrelia* species in ticks that have bitten humans may reveal information about their previous hosts. *B. afzelii* has frequently been associated with rodents and *B. garinii* and *B. valaisiana* with birds (Hanincova *et al.*, 2003a; Hanincova *et al.*, 2003b; Kurtenbach *et al.*, 2002b). *B. burgdorferi sensu stricto* is a species that has the ability to persist in a wide range of hosts, and *B. lusitaniae* has been associated with lizards (Dsouli *et al.*, 2006; Kurtenbach *et al.*, 2002b; Richter & Matuschka, 2006). To investigate such associations for the *Borrelia* species detected in the TBD STING-study, one could analyze the previous blood-meal of the *Borrelia*-infected ticks, in which host DNA can be identified, as discussed earlier.

How is the Borrelia *load (the number of* Borrelia *cells) in a tick that feeds on a human affected by the duration time of tick feeding?*

The number of *Borrelia* cells in infected *I. ricinus* ticks may range from fewer than ten, close to detection limit, to more than a million (Fig. 3, **III**). After 36 hours of feeding on humans, adult female ticks infected with either *B. afzelii* or *B. garinii* contained a significantly lower *Borrelia* load compared to adult female ticks that had fed shorter than 36 hours (Fig. 5, **III**). The reason for this is unknown, but when we first observed the difference, we questioned whether the lower *Borrelia* loads, found in the adult female ticks that had fed longer than 36 hours, represented PCR inhibition because of the greater volume of blood present. However, when we evaluated our LUX real-time PCR assay, no sign of inhibition was noticed (**III**). One can therefore speculate whether the lower *Borrelia* loads, found in the adult female ticks that had fed longer than 36 hours, represented a transmission of the *Borrelia* bacteria from tick to human. But to confirm that, one would have to investigate if any *Borrelia* bacteria actually were transmitted to the bitten person. In contrast to our findings, i.e.

a lower *Borrelia* load in ticks with longer feeding-time, Piesman and co-workers reported a sixfold higher *Borrelia* load of *B. burgdorferi sensu stricto* in *I. scapularis* nymphs that had fed for two days on mice compared to non-fed ticks (from a total of 998 per tick to 5,884) (Piesman et al., 2001). In our study we investigated a different tick species, different *Borrelia* species and different kinds of hosts. Studies by Kahl and co-workers, and by Crippa and co-workers have shown that one cannot generalize the study of one *B. burgdorferi* senu lato species to another. For instance, the transmission dynamics of *B. burgdorferi sensu stricto* is different from that of *B. afzelii*, which is transmitted earlier to the host than *B. burgdorferi sensu stricto* (Crippa et al., 2002; Kahl et al., 1998). One could also speculate whether the lower *Borrelia* loads, found in adult female ticks that had fed longer than 36 hours, represented a higher degradation of *Borrelia* bacteria in those ticks. These ticks had ingested a larger volume of blood, thus a higher level of innate immune cells which are capable of eliminating the *Borrelia* bacteria. Since nymphs are smaller than adult females, they ingest a smaller volume of blood, thus a lower level of innate immune cells. This could explain why we did not observed a similar reduction of the *Borrelia* loads in nymphs (>36h) as we did for the adult female ticks (>36h).

The dynamics of the *Borrelia* load in a feeding tick is probably dependent on many factors such as duration of tick feeding, tick species, life stage of the tick, *Borrelia* species, initial number of *Borrelia* bacteria in the tick before it begins to feed, and species of host. We analyzed the *Borrelia* load in *I. ricinus* ticks that had fed on humans. Knowledge about the *Borrelia* load in such ticks should be valuable when effectiveness of vaccine candidates, that are designed to kill the *Borrelia* bacteria while they are still inside a feeding tick, is to be evaluated. In our study, we recorded that ticks infected with *B. miyamotoi*, contained significantly more *Borrelia* cells compared to

ticks infected with other species (Fig. 3, **III**). One should, therefore, evaluate if the level of antibodies produced by a *Borrelia* vaccinated person, is enough to eradicate a higher quantity of *B. miyamotoi* bacteria in the ticks.

Another benefit to quantify the *Borrelia* load in ticks is that the data can be used to study if ticks infected with higher *Borrelia* load play a more important role in maintaining *Borrelia* spp. in nature than ticks infected with lower *Borrelia* load. However, an important limit of using real-time PCR methods to detect and quantify the *Borrelia* bacteria is that it does not differentiate between live bacteria and dead remnants.

Part 3. The interactions between *Borrelia* bacteria and humans – results and discussion (paper IV)

In an effort to examine the clinical outcome (prognosis) and the serological response after a bite by a *Borrelia*-infected tick, we collected and analyzed serum samples from tick-bitten persons (n = 1546) between 2008 and 2009. The clinical outcome and the serological response of the persons were related to their answers to the questionnaires and to their medical records, if they visited health care during the three-month study period. The clinical outcome and the serological response of the tick-bitten persons were also related to the infection status of the ticks, to the *Borrelia* load in the infected ticks, and to the feeding duration of the ticks.

What are the clinical outcome (prognosis) and the serological response after a bite by a Borrelia-*infected tick?*

The incidence of LB after a bite by a *Borrelia*-infected tick is low. Of all tick bites, 2% (32/1546) of the participants developed LB. Barely 4% (16/428) of the participants that were bitten by *Borrelia*-infected ticks developed LB (Fig. 2, **IV**).

EM was the most prevalent symptom among the LB patients (85%, 27/32, Table 3, **IV**). EM is a local manifestation and is, in Sweden and in the rest of Europe, most often caused by *B. afzelii* (Ornstein *et al.*, 2001; Stanek *et al.*, 2011). About 11% of all tick bites, recorded by the TBD STING-study participants, were caused by ticks that contained *B. afzelii* (Table 1, **III**). Of the EM patients in the TBD-STING study, the EM manifestation evolved at the same location as the tick bite (Table

3, **IV**), and the majority of those ticks (5/6) were infected with *B. afzelii*. This strongly indicates that these PCR-positive ticks are responsible for transmitting the *Borrelia* bacteria to the bitten EM patients. But to confirm that, one would have to detect the same *Borrelia* strain in both the tick and in the patient.

Only two of the 1546 tick-bitten participants were diagnosed with NB (Table 3, **IV**). In Sweden and in the rest of Europe, NB is most often caused by *B. garinii* (Ornstein *et al.*, 2001; Stanek *et al.*, 2011), and about 5% of the tick bites in the TBD-STING study were caused by ticks that contained this species (Table 1, **III**). Not surprisingly, one of the two NB patients in the TBD STING-study was bitten by a tick that contained *B. garinii*. A CSF sample taken from this NB patient was analyzed for the presence of *Borrelia* bacteria with our real-time PCR assay but no *Borrelia* was found (unpublished results). If the same *Borrelia* strain had been found in the CSF as in the tick, it would have permitted interesting gene expression studies of the *Borrelia* bacteria: Information regarding up- and downregulations of borrelial proteins in different environments is fundamental to identify and to understand immune evasion mechanisms of the *Borrelia* bacteria. The other NB patient in the TBD STING-study was bitten by a tick that was *Borrelia*-negative with our real-time PCR assay, but this NB patient also contracted other tick bites (n = 5) during the three-month study period, which may explain the *Borrelia* infection. These ticks will be analyzed.

One of the 1546 tick-bitten participants was diagnosed with BL (Table 3, **IV**). BL is a rare manifestation among LB patients, and when it manifests it usually appears on earlobes, nipples or in scrotum area, usually close to the tick bite (Strle *et al.*, 1992). On this BL patient, the manifestation evolved at the earlobe, the same location as the tick bite (Table 3, **IV**). The tick was also infected with *B. afzelii*, a species

that is usually the cause of BL (Maraspin *et al.*, 2002; Picken *et al.*, 1997). Interestingly, this BL patient developed the manifestation during the three-month study period, but was seronegative both at inclusion time and three months later. After the three-month study period, however, a new serum sample was taken from the patient by a clinician and both IgM and IgG against *B. burgdorferi* sensu lato were detected, as stated in the medical records of the patient. The reason why this BL patient was seronegative during the three-month study period is unknown, but since BL is considered as a local manifestation it could be due to a delay in the antibody production of the patient. It could also be due to methodological differences, the clinician used other antigens to detect the presence of antibodies than those that were applied in the TBD STING-study.

Three months after a tick bite, 23% (349/1546) of the participants reported non-specific symptoms possibly associated with LB (Table 1, **IV**). Two of these were clinically diagnosed with LB (Table 3, **IV**). According to their medical records, both of them suffered from myalgia/arthralgia and both of them seroconverted. When we analyzed their sera, taken at inclusion and three months later, one of them seroconverted but the other one did not (Table 3, **IV**). However, the non-seroconverted participant was seropositive in both sera.

Besides the 2% (32/1546) who were clinically diagnosed with LB, 2.5% (39/1546) seroconverted without an LB diagnose (Fig. 2, **IV**). It is not rare that individuals, exposed to *B. burgdorferi* sensu lato bacteria, are asymptomatic (Ekerfelt *et al.*, 2001; Fahrer *et al.*, 1991b). The natural history of asymptomatic *Borrelia* infection is unknown, but infection with non-invasive *Borrelia* strains as well as inter-individual differences in the immune response against *B. burgdorferi* sensu lato, have been proposed as explanations (Sjowall *et al.*, 2005; Wormser *et al.*, 2001).

In Sweden as well as on the Åland Islands, there is no LB notification system and thus, the incidence of LB is unknown. However, according to the epidemiological investigation of LB that was conducted in southern Sweden in 1995, there might be an annual incidence of 69 LB cases per 100,000 inhabitants (Berglund *et al.*, 1995). An extrapolation of this incidence to the entire Swedish population indicates that there might be around 6,000 new LB cases in Sweden every year. This figure of estimation is frequently told by media. One should bear in mind that Berglund's study was carried out 20 years ago, and the LB incidence today may be different. Since that study was conducted, the distribution area of ticks in Sweden has increased along with density of the tick population (Jaenson *et al.*, 2009). This has lead to an increased rate of tick bites among the Swedish inhabitants and consequently an increased rate of LB cases. In the TBD STING-study, the incidence of LB in a population bitten by ticks was investigated (**IV**). Extrapolation of the LB incidence of 2% to the 1.3 million Swedish individuals (18% of the entire population) that were estimated to have contracted tick bites during 2005 (Boehringer-Ingelheim, 2006), results in an calculated incidence of 26,000 LB cases every year, which is four times higher than previously estimated (Berglund *et al.*, 1995). However, several difficulties are associated with this kind of estimations. First of all, the tick bite incidence among people is probably not constant between years. Secondly, the TBD STING-study participants are probably not representative for the entire population of the study regions. It may rather represent a group of people that are particularly vulnerable to LB, as discussed below.

In other European countries, the age distribution of the LB disease has been reported to be bimodal where children (5-9 years), and persons aged 50-64 years are particular vulnerable (Hubalek, 2009). Reports also indicate that women rather than men are more susceptible to symptomatic *Borrelia* infections (Bennet *et al.*, 2007; Fulop &

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Poggensee, 2008; Strle *et al.*, 2013). The typical TBD STING-study participant was in average 63 years old and woman (65% women, and 35% men, **IV**). Thirty-nine percentages of all participants reported a medical history of LB (Table 2, **IV**), 42% of the women and 35% of the men. Between 32 and 35% of the participants from the Swedish regions reported a medical history of LB, except in Northern Sweden, where only one participant out of 12 reported such history (Table 2, **IV**). On the Åland Islands, on the other hand, 50% reported a medical history of LB. The fact that "older" people with a history of LB were more willing to participate in the study, than younger persons with no history of LB, indicates that there might be a selection bias of individuals. This may have influenced the LB incidence rate among the tick-bitten participants in the TBD STING-study: A previous *Borrelia* infection may lead to the production of antibodies and memory cells, which are intended to be protective against re-infection. Based on this argument, the LB incidence after a tick bite found in the TBD STING-study (2%, **IV**) may therefore be underestimated. However, far from all *Borrelia* infections in humans leads to an antibody production. About 30% of those who gets antibiotic treatment early during the infection, e.g. when the EM mark manifest, resolves the *Borrelia* infection before the immune system starts to produce antibodies (Aberer & Schwantzer, 2012). And even if it starts to produce antibodies as a response to a *Borrelia* infection, it is no assurance that the antibodies are protective: The genetic diversity of surface proteins and their surface-exposed residues are highly variable within the same *Borrelia* species (Eicken *et al.*, 2001; Wang *et al.*, 1999c). The LB incidence after a tick bite (2%) may therefore instead be overestimated. A great proportion, sometimes more than half, of the participants reported a history of LB, which indicates that the studied population may be particularly susceptible to *Borrelia* infections.

Are there gender- and geographical differences in the Borrelia *seroprevalence among tick-bitten persons?*

Of all the TBD STING-study participants, about 20% was seropositive for IgG anti-flagellum antibodies at inclusion time (Table 2, **IV**). A lower proportion of the women (16%, 156/1002) than men (27%, 147/544) was seropositive. This may indicate a gender difference in contracting tick bites, i.e. men rather than women may contract more tick bites. However, a study on the risk for people to be infested by ticks in southernmost Sweden found no significant difference between men and women (Stjernberg & Berglund, 2002). A similar study on the western coast of Norway showed that women >50 years actually contracted more tick bites than similarly aged men and younger women (Hjetland *et al.*, 2013). In addition, a higher proportion of the women in the TBD STING-study contracted more tick bites during the study period than the men did (unpublished results). Therefore, exposure to tick bites may not explain the gender difference in the *Borrelia*-seroprevalence. Instead, it may be explained by gender differences in the serological and clinical response to *Borrelia* infections. It has been shown in the Swedish population (aged ≥ 45 years) that more women than men develop LB (Berglund *et al.*, 1995). A female predominance of LB (i.e. EM) has also been reported in other European epidemiologic studies (Mehnert & Krause, 2005; Stanek *et al.*, 1987; Strle *et al.*, 2002). It has also been shown that women over 44 years with EM manifestations are more often reinfected with *Borrelia* after antibiotic treatment, compared to similarly aged men with EM (Bennet & Berglund, 2002). The reason why "older" women are particular susceptible to LB infections compared to similarly aged men and younger women is unknown, but differences in biological mechanisms may be involved. In general, estrogen has a stimulatory effect on the immune system whereas testosterone acts as a suppressor. When "older" women enter menopause their

levels of estrogen decrease and thereby the stimulatory effect may diminish, leading to an altered immune status (Olsen & Kovacs, 1996). In the TBD STING-study, a medical history of LB was more frequently reported by women (42%) than men (35%), but a lower proportion of women (16%) than men (27%) were seropositive (**IV**). This could be due to gender-dependent behavioral differences in seeking health care with respect to LB. As mentioned before, antibiotic treatment early during the infection, e.g. when the EM mark manifest, resolve the *Borrelia* infection before the immune system starts to produce antibodies (Aberer & Schwantzer, 2012). However, we have not found any data in literature indicating gender-dependent behavioral differences in seeking health care with respect to LB.

Different regions may have different tick burdens. In some regions there is a small risk of contracting tick bites and thus, a small risk of contracting *Borrelia* infections, other regions may be highly endemic. Of all the TBD STING-study participants, about 20% was seropositive for IgG anti-flagellum antibodies at inclusion time (Table 2, **IV**). A higher proportion of participants from the Åland Islands (23%) were seropostive compared to South Central Sweden (17%) and to Southernmost Sweden (18%). In Northern Sweden, 5 out of 12 participants (42%) were seropositive. The frequency of exposure to tick bites may, at least partly, explain these observations. On the Åland Islands it is well-known that ticks are abundant and tick bites are commonly reported by the inhabitants (Wahlberg, 1990). In addition, the annual LB incidence on the Åland Islands is about 1000/100,000 inhabitants (years 2000-2012) (THI, 2013), which indicate that it is a hyperendemic region. All this together may explain why the seroprevalence was higher among the participants from the Åland Islands compared to the participants from South Central Sweden and from Southernmost Sweden. The seroprevalence in the TBD STING-study (20%) is within the range of those reported from other Europe-

an studies: in healthy blood donors the seroprevalence ranged between 2 and 24% (Ekerfelt *et al.*, 2001; Fahrer *et al.*, 1991a; Gustafson *et al.*, 1993a; Hristea *et al.*, 2001; Tjernberg *et al.*, 2007; Tomao *et al.*, 2005); in people living in endemic regions between 7 and 29% (Berglund & Eitrem, 1993; Carlsson *et al.*, 1998; Gustafson *et al.*, 1993b; Werner *et al.*, 2001); and for people in high-risk groups (outdoor workers, orienteers and hunters) between 9 and 54% (Cetin *et al.*, 2006; Fahrer *et al.*, 1991a; Gustafson *et al.*, 1993a; Hristea *et al.*, 2001; Tomao *et al.*, 2005).

Which factors may facilitate Borrelia *transmission from tick to human and give rise to LB?*

There are probably many factors involved in the *Borrelia* transmission from tick to human which may contribute to LB: Life stage of tick, attachment site on the body, duration of tick feeding, species or strain of the *Borrelia* bacteria, the *Borrelia* load (the number of *Borrelia* cells) in the tick, the immunological response of the tick-bitten human among many other factors.

We observed that most tick bites on humans were caused by *I. ricinus* nymphs, and they usually took place on the legs (**I**). However, ticks attached to the genitals or to the head and neck were, in general, detected and removed later. This means that ticks attached to these sites fed for a longer time before they are detected, which would be more permissive for a potential *Borrelia* transmission (Crippa *et al.*, 2002; Kahl *et al.*, 1998). It has earlier been shown that tick-bitten people that remove ticks later than 24 hours of tick attachment, as self-estimated by the bitten persons, are more likely to develop localized and systemic symptoms (Tijsse-Klasen *et al.*, 2011), probably due to injected tick salivary gland proteins and/or due to transmitted

pathogens. We observed that participants who contracted a *Borrelia* infection (seroconversion) during the three-month study period detected and removed "their" *Borrelia*-infected ticks later (median 58 hours) than non-infected participants bitten by *Borrelia*-infected ticks (median 29 hours, **IV**). This is the first time such correlation between the duration of tick feeding, estimated by the scutal and coxal indices, and the serological response in tick-bitten humans is shown. Although the risk of developing a *Borrelia* infection after a tick bite is very low, an early finding and a prompt removal may reduce the risk of *Borrelia* transmission and the risk of developing LB.

For a *Borrelia* transmission to occur from a tick to a human, the tick must carry viable *Borrelia* bacteria. In the TBD STING-study, 26% of ticks that had bitten humans were infected with *Borrelia* bacteria, and a total of seven *Borrelia* species were detected (**II**, **III**). Different strains of these *Borrelia* species have previously been linked to clinical samples of LB. In the TBD STING-study, we cannot elucidate if the *Borrelia* bacteria, detected in the removed ticks, are viable due to the detection methods used (discussed earlier). One should also bear in mind that the *Borrelia*-status of a removed tick does not explain the infection risk of a person bitten by a tick. A *Borrelia*-negative tick may already have transmitted its *Borrelia* bacteria to the bitten person, and a *Borrelia*-positive tick may have not.

The *Borrelia* load in a tick that bites a human may play an important role in the transmission of *Borrelia* bacteria from tick to human. It is possible that ticks infected with few *Borrelia* cells are unable to transmit the *Borrelia* bacteria (de Silva *et al.*, 1999). Wang and co-workers speculated that low numbers of *Borrelia* cells (≤ 300) found in unfed field-collected ticks may represent a transmission threshold (Wang *et al.*, 2003). Based on this, one may also speculate that "heavily" infected ticks may play a greater role in the *Borrelia* trans-

mission. In the TBD STING-study, the *Borrelia* load in the ticks did not explain the risk of infection (**IV**), i.e. the *Borrelia* load in ticks did not differ significantly between those removed by participants who later seroconverted (median 2800 *Borrelia* cells, IQR 500–17000), and those removed by participants who did not seroconvert (median 1600, IQR 100-16000). Our results do not allow a calculation of the amount of *Borrelia* cells transmitted by a feeding tick since we have only estimates of the number of *Borrelia* cells present in the tick at discrete points in time. It has earlier been shown that there is a correlation between clinical symptoms and the *Borrelia* load in human tissues (O'Rourke *et al.*, 2013): The *Borrelia* load was significantly higher among EM patients with systemic symptoms than without, and was significantly higher for biopsies retrieved from patients with EM lesions with central clearing. However, the magnitude of the initial *Borrelia* load that is transmitted to humans could also play an important role for the onset of LB manifestations, as well as for the development of antibodies and the resolution of infection. To investigate this one would have to quantify the *Borrelia* cells at the site of the tick bite and follow the clinical and serological response of the tick-bitten persons over time.

Summary of the findings

Part 1: The interactions between humans and ticks

Most of the participants were exposed to tick bites between April and November, but temporal and spatial differences in exposure to the different life stages of *I. ricinus* ticks was found. The majority of the tick bites were caused by nymphs and most tick bites took place on the legs. The time it takes for a person to detect and remove an attached tick appeared to be influenced by the site of tick attachment and by the gender and age of the bitten person. The majority of the tick-bitten participants removed "their" ticks later than 24 hours of tick attachment.

Part 2: The interactions between ticks and *Borrelia* bacteria

Every fourth tick that had bitten the participants was infected with *Borrelia* bacteria. Six species of the *B. burgdorferi* sensu lato complex and the TBRF-causing species *B. miyamotoi* were detected. Some of the species were detected for the first time in the studied areas. The adult ticks that had fed for more than 36 hours contained a lower number of *Borrelia* cells than the adult ticks that had fed less than 36 hours.

Part 3: The interactions between *Borrelia* bacteria and humans

Two percent of all tick-bitten participants and 4% of the participants bitten by *Borrelia*-infected ticks were diagnosed with LB, where EM was the most common LB manifestation. More than 2% of the participants seroconverted without an LB diagnose. A correlation between seroconversion and duration time of tick attachment was found but the *Borrelia* load in the tick did not explain the risk of infection. The

seroprevalence among the tick-bitten participants varied between genders as well as between the studied geographical areas.

Concluding remarks

The overall aims of the thesis were to increase our understanding of the epidemiology of the *Borrelia* bacteria, and the ecology of ticks, as well as to increase our understanding of the clinical situation of LB. Furthermore, to provide information that can help reduce the number of tick bites and the number of LB cases in tick-infested areas. In order to achieve these goals, three parts (**1-3**) of the interactions between humans, ticks, and *Borrelia* bacteria were thoroughly investigated:

Part 1: The interactions between humans and ticks
The knowledge concerning the interactions between humans and ticks is limited. To improve our understanding of the seasonal tick infestation pattern on humans, we studied temporal and spatial differences in exposure to different life stages of *I. ricinus*. Such information may be used to raise public awareness about when during the year tick bites among humans may occur. This could potentially lead to a reduced number of tick bites and, consequently, to a reduced number of tick-borne infections among people. We also studied the site of tick attachment on the human body and when such ticks generally are detected and removed in relation to host age and gender. This kind of information is valuable for the development of prophylactic methods against tick bites and to provide relevant advice to people on how to avoid or reduce the risk of tick bites.

Part 2: The interactions between ticks and *Borrelia* bacteria

Most of our knowledge of the *Borrelia* bacteria in ticks originates from studies on tick samples from vegetation or tick samples from animals. To obtain a deeper knowledge of the *Borrelia* bacteria epidemiology, we studied the prevalence, the species diversity and the *Borrelia* load in ticks that had bitten humans. Information about the prevalence and distribution of *Borrelia* species in ticks that bite humans is important for risk assessment analysis and to our understanding of the *Borrelia* circulation within the natural cycle between ticks and hosts. Information about the *Borrelia* species that circulates among ticks that bite humans should also be of great value when diagnostic tools for LB diagnosis are developed. Studies on the dynamics of the *Borrelia* load in ticks that have fed blood on humans may improve our understanding of how *Borrelia* spirochetes are transmitted from tick to humans and how to prevent such transmission.

Part 3: The interactions between *Borrelia* bacteria and humans

In Sweden, no mandatory LB notification system exists and the awareness of the clinical situation of LB is poor. To increase our knowledge of this issue, we studied the seroprevalence of *Borrelia* antibodies and the incidence of *Borrelia* infections among recently tick-bitten persons from geographically different areas. Studies on the seroprevalence of *Borrelia* among people together with their history of LB may provide important data on the occurrence of symptomatic and asymptomatic *Borrelia* infections. This may define population groups that are susceptible to developing symptomatic *Borrelia* infections, which could be useful to assist prevention strategies. Knowing the incidence of LB in a population is important for a number of reasons. It allows quantification of the magnitude of the LB problem, facilitating appropriate health care resource allocation and

prediction of future resource requirements. It may allow an estimation of the economic as well as other costs of the LB condition to the given population. Knowing the incidence of *Borrelia* infections in a population may also help to identify environmental influences that lead to the development of infection: In this study, we investigated if the duration time of tick attachment and *Borrelia* load in ticks influenced the serological response of the bitten persons. Such information should be a great value for risk assessment of LB.

Future perspectives

In this thesis, we studied the interactions between ticks, humans and *Borrelia* bacteria, but other issues need to be addressed as well (Figure 8). In 2008 and 2009, we studied the temporal and spatial tick infestation patterns on humans in four different geographical areas in Sweden and on the Åland Islands. However, it is also important to investigate the tick infestation patterns on humans in other areas and if the infestation patterns change over time due to possible climate changes and/or changes in the density and distribution of important maintenance hosts. Such information would be valuable when predicting future "tick bite" scenarios. In addition, identification of important *Borrelia* reservoir hosts could be useful to assist prevention strategies for *Borrelia* infections, e.g. to lower the circulation of *Borrelia* bacteria in nature by controlling such hosts.

In this thesis, we focused our studies on one of the two major groups of people that are at a particular risk of contracting LB, i.e. older persons (median age of 63 years). It would also be relevant to study the other high-risk group, i.e. children (< 10 years), and "their" tick infestation pattern and their clinical and serological response to tick bites.

We studied the prevalence and distribution of *Borrelia* species as well as the *Borrelia* load in ticks that had bitten humans, where 2% was clinically diagnosed with LB. To date, we do not know if the *Borrelia* strain detected in the tick, is the same strain that caused the infection in the tick-bitten human. Comparison of gene expression profiles of the same *Borrelia* strain present in both tick and in clinical specimen remains the "holy grail" of gene expression measurements with regard to transmission dynamics of the *Borrelia* bacteria.
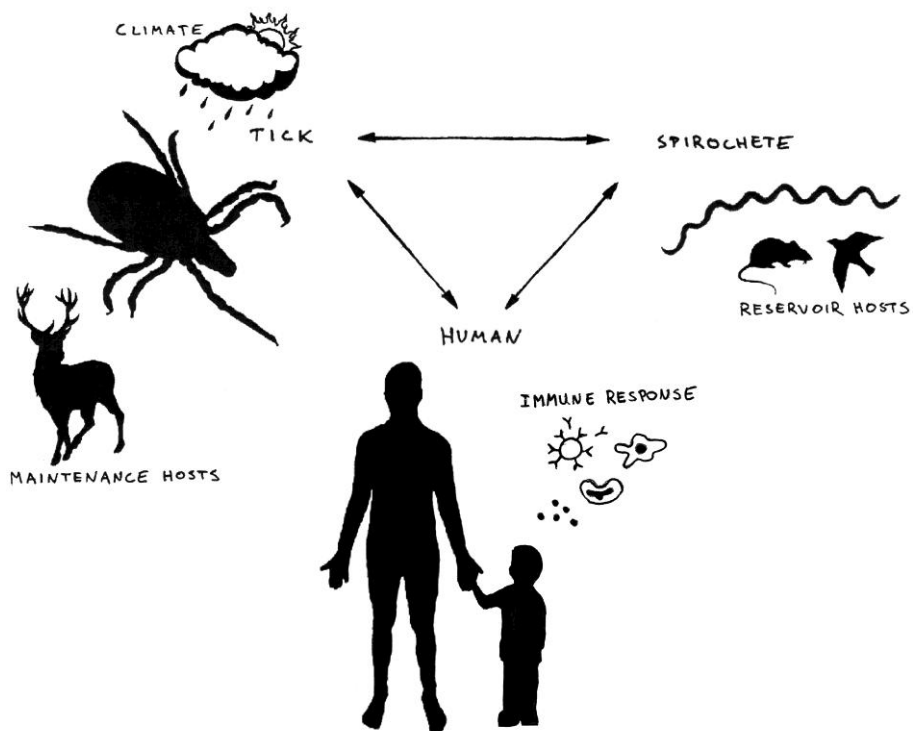
Figure 8. Schematic outline of the studied interactions and factors that need to be further investigated.

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References

Aberer, E. & Duray, P. H. (1991). Morphology of Borrelia burgdorferi: structural patterns of cultured borreliae in relation to staining methods. J Clin Microbiol 29, 764-772.

Aberer, E. & Schwantzer, G. (2012). Course of Antibody Response in Lyme Borreliosis Patients before and after Therapy. ISRN Immunology 2012, 4.

Afzelius, A. (1910). Verhandlungen der dermatologischen Gesellschaft zu Stockholm, 28 Oktober 1909. 101, 405-406.

Afzelius , A. (1921). Erythema migrans. Acta Derm Venereol 2, 120-125.

Aguero-Rosenfeld, M. E., Wang, G., Schwartz, I. & Wormser, G. P. (2005). Diagnosis of lyme borreliosis. Clin Microbiol Rev 18, 484-509.

Alitalo, A., Meri, T., Lankinen, H., Seppala, I., Lahdenne, P., Hefty, P. S. & other authors (2002). Complement inhibitor factor H binding to Lyme disease spirochetes is mediated by inducible expression of multiple plasmid-encoded outer surface protein E paralogs. J Immunol 169, 3847-3853.

Anderson, J. F. & Magnarelli, L. A. (1993). Epizootiology of Lyme disease-causing borreliae. Clin Dermatol 11, 339-351.

Anguita, J., Ramamoorthi, N., Hovius, J. W., Das, S., Thomas, V., Persinski, R. & other authors (2002). Salp15, an ixodes scapularis salivary protein, inhibits CD4(+) T cell activation. Immunity 16, 849-859.

Asbrink, E., Hovmark, A. & Hederstedt, B. (1984). The spirochetal etiology of acrodermatitis chronica atrophicans Herxheimer. Acta Derm Venereol 64, 506-512.

Asbrink, E., Hovmark, A. & Olsson, I. (1986). Clinical manifestations of acrodermatitis chronica atrophicans in 50 Swedish patients. Zentralbl Bakteriol Mikrobiol Hyg A 263, 253-261.

Asbrink, E. & Hovmark, A. (1988). Early and late cutaneous manifestations in Ixodes-borne borreliosis (erythema migrans borreliosis, Lyme borreliosis). Ann N Y Acad Sci 539, 4-15.

Asch, E. S., Bujak, D. I., Weiss, M., Peterson, M. G. & Weinstein, A. (1994). Lyme disease: an infectious and postinfectious syndrome. J Rheumatol 21, 454-461.

Auwaerter, P. G. (2007). Point: antibiotic therapy is not the answer for patients with persisting symptoms attributable to lyme disease. Clin Infect Dis 45, 143-148.

Bacon, R. M., Kugeler, K. J., Griffith, K. S. & Mead, P. S. (2007). Lyme disease - United States, 2003-2005 (Reprinted from MMWR, vol 56, pg 573-576, 2007). Jama-J Am Med Assoc 298, 278-279.

Bannwarth, A. (1941). Chronische lymphocytäre Meningitis, entzundliche Polyneuritis und Rhematismus. Arch Psychiatr Nervenkrank, 161-185.

Barbour, A. G. (1984). Isolation and cultivation of Lyme disease spirochetes. Yale J Biol Med 57, 521-525.

Barbour, A. G. & Hayes, S. F. (1986). Biology of Borrelia species. Microbiol Rev 50, 381-400.

Barbour, A. G., Bunikis, J., Travinsky, B., Hoen, A. G., Diuk-Wasser, M. A., Fish, D. & other authors (2009). Niche partitioning of Borrelia burgdorferi and Borrelia miyamotoi in the same tick vector and mammalian reservoir species. Am J Trop Med Hyg 81, 1120-1131.

Barker, S. C. & Murrell, A. (2004). Systematics and evolution of ticks with a list of valid genus and species names. Parasitology 129 Suppl, S15-36.

Barsic, B., Maretic, T., Majerus, L. & Strugar, J. (2000). Comparison of azithromycin and doxycycline in the treatment of erythema migrans. Infection 28, 153-156.

Bennet, L. & Berglund, J. (2002). Reinfection with Lyme borreliosis: a retrospective follow-up study in southern Sweden. Scand J Infect Dis 34, 183-186.

Bennet, L., Fraenkel, C. J., Garpmo, U., Halling, A., Ingman, M., Ornstein, K. & other authors (2006). Clinical appearance of erythema migrans caused by Borrelia afzelii and Borrelia garinii--effect of the patient's sex. Wien Klin Wochenschr 118, 531-537.

Bennet, L., Stjernberg, L. & Berglund, J. (2007). Effect of gender on clinical and epidemiologic features of Lyme borreliosis. Vector Borne Zoonotic Dis 7, 34-41.

Berglund, J. & Eitrem, R. (1993). Tick-borne borreliosis in the archipelago of southern Sweden. Scand J Infect Dis 25, 67-72.

Berglund, J., Eitrem, R., Ornstein, K., Lindberg, A., Ringer, A., Elmrud, H. & other authors (1995). An epidemiologic study of Lyme disease in southern Sweden. N Engl J Med 333, 1319-1327.

Berglund, J., Eitrem, R. & Norrby, S. R. (1996). Long-term study of Lyme borreliosis in a highly endemic area in Sweden. Scand J Infect Dis 28, 473-478.

Boehringer-Ingelheim (2006). Drygt 1,3 miljoner svenskar blev bitna av fästing senaste säsongen [Press release]. Retrieved from http://feed.ne.cision.com/wpyfs/00/00/00/00/00/07/A1/21/release.html , pp. 1-3. Stockholm.

Bratton, R. L., Whiteside, J. W., Hovan, M. J., Engle, R. L. & Edwards, F. D. (2008). Diagnosis and treatment of Lyme disease. Mayo Clin Proc 83, 566-571.

Brorson, O. & Brorson, S. H. (1997). Transformation of cystic forms of Borrelia burgdorferi to normal, mobile spirochetes. Infection 25, 240-246.

Brorson, O. & Brorson, S. H. (1998). A rapid method for generating cystic forms of Borrelia burgdorferi, and their reversal to mobile spirochetes. APMIS 106, 1131-1141.

Buchwald, A. (1883). Ein Fall von diffuser idiopathischer Haut-Atrophie. Archiv für Dermatologie und Syphilis, 553-556.

Bunikis, J., Tsao, J., Garpmo, U., Berglund, J., Fish, D. & Barbour, A. G. (2004). Typing of Borrelia relapsing fever group strains. Emerg Infect Dis 10, 1661-1664.

Burckhardt, J. (1911). Zur Frage der Follikel- und Keimzentrenbildung in der Haut. Frankfurter Zeitschrift für Pathologie, 352-359.

Burdash, N. & Fernandes, J. (1991). Lyme borreliosis: detecting the great imitator. J Am Osteopath Assoc 91, 573-574, 577-578.

Burgdorfer, W., Barbour, A. G., Hayes, S. F., Benach, J. L., Grunwaldt, E. & Davis, J. P. (1982). Lyme disease-a tick-borne spirochetosis? Science 216, 1317-1319.

Burgdorfer, W. (1984). Discovery of the Lyme disease spirochete and its relation to tick vectors. Yale J Biol Med 57, 515-520.

Burkot, T. R., Piesman, J. & Wirtz, R. A. (1994). Quantitation of the Borrelia burgdorferi outer surface protein A in Ixodes scapularis: fluctuations during the tick life cycle, doubling times, and loss while feeding. J Infect Dis 170, 883-889.

Busch, U., Hizo-Teufel, C., Bohmer, R., Fingerle, V., Rossler, D., Wilske, B. & other authors (1996). Borrelia burgdorferi sensu lato strains isolated from cutaneous Lyme borreliosis biopsies differentiated by pulsed-field gel electrophoresis. Scand J Infect Dis 28, 583-589.

Bäfverstedt, B. (1943).Über Lymphadenosis benigna cutis. Eine klinische und pathologisch-anatomische Studie. Stockholm: P.A. Norstedt & Söner.

Carlsson, S. A., Granlund, H., Nyman, D. & Wahlberg, P. (1998). IgG seroprevalence of Lyme borreliosis in the population of the Aland Islands in Finland. Scand J Infect Dis 30, 501-503.

Casjens, S. (2000). Borrelia genomes in the year 2000. J Mol Microbiol Biotechnol 2, 401-410.

Casjens, S., Palmer, N., van Vugt, R., Huang, W. M., Stevenson, B., Rosa, P. & other authors (2000). A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete Borrelia burgdorferi. Mol Microbiol 35, 490-516.

CDC (1995). Centers for Disease Control and Prevention. Recommendations for test performance and interpretation from the Second National Conference on Serologic Diagnosis of Lyme Disease. MMWR Morb Mortal Wkly Rep 44, 590-591.

CDC (2013). Centers for Disease Control and Prevention. Three sudden cardiac deaths associated with lyme carditis - United States, november 2012-july 2013. MMWR Morb Mortal Wkly Rep 62, 993-996.

CDC (2014). Centers for Disease Control and Prevention. What you need to know about Borrelia miyamotoi, a newly described human pathogen. Retrieved from http://www.cdc.gov/ticks/miyamotoi.html. 2014-03-14.

Cerar, T., Ruzic-Sabljic, E., Glinsek, U., Zore, A. & Strle, F. (2008). Comparison of PCR methods and culture for the detection of Borrelia spp. in patients with erythema migrans. Clin Microbiol Infect 14, 653-658.

Cetin, E., Sotoudeh, M., Auer, H. & Stanek, G. (2006). Paradigm Burgenland: risk of Borrelia burgdorferi sensu lato infection indicated by variable seroprevalence rates in hunters. Wien Klin Wochenschr 118, 677-681.

Chmielewski, T. & Tylewska-Wierzbanowska, S. (2010). Interactions between Borrelia burgdorferi and mouse fibroblasts. Pol J Microbiol 59, 157-160.

Cinco, M., Cini, B., Murgia, R., Presani, G., Prodan, M. & Perticarari, S. (2001). Evidence of involvement of the mannose receptor in adhesion of Borrelia burgdorferi to monocyte/macrophages. Infect Immun 69, 2743-2747.

Clark, R. P. & Hu, L. T. (2008). Prevention of lyme disease and other tick-borne infections. Infect Dis Clin North Am 22, 381-396, vii.

Coburn, J., Magoun, L., Bodary, S. C. & Leong, J. M. (1998). Integrins alpha(v)beta3 and alpha5beta1 mediate attachment of lyme disease spirochetes to human cells. Infect Immun 66, 1946-1952.

Coleman, J. L., Sellati, T. J., Testa, J. E., Kew, R. R., Furie, M. B. & Benach, J. L. (1995). Borrelia burgdorferi binds plasminogen, resulting in enhanced penetration of endothelial monolayers. Infect Immun 63, 2478-2484.

Coleman, J. L., Gebbia, J. A., Piesman, J., Degen, J. L., Bugge, T. H. & Benach, J. L. (1997). Plasminogen is required for efficient dissemination of B. burgdorferi in ticks and for enhancement of spirochetemia in mice. Cell 89, 1111-1119.

Colli, C., Leinweber, B., Mullegger, R., Chott, A., Kerl, H. & Cerroni, L. (2004). Borrelia burgdorferi-associated lymphocytoma cutis: clinicopathologic, immunophenotypic, and molecular study of 106 cases. J Cutan Pathol 31, 232-240.

Connolly, S. E. & Benach, J. L. (2005). The versatile roles of antibodies in Borrelia infections. Nat Rev Microbiol 3, 411-420.

Crippa, M., Rais, O. & Gern, L. (2002). Investigations on the mode and dynamics of transmission and infectivity of Borrelia burgdorferi sensu stricto and Borrelia afzelii in Ixodes ricinus ticks. Vector Borne Zoonotic Dis 2, 3-9.

Czerkinsky, C., Andersson, G., Ekre, H. P., Nilsson, L. A., Klareskog, L. & Ouchterlony, O. (1988). Reverse ELISPOT assay for clonal analysis of cytokine production. I. Enumeration of gamma-interferon-secreting cells. J Immunol Methods 110, 29-36.

Daniels, T. J., Fish, D. & Falco, R. C. (1989). Seasonal activity and survival of adult Ixodes dammini (Acari: Ixodidae) in southern New York State. J Med Entomol 26, 610-614.

Daniels, T. J., Fish, D. & Schwartz, I. (1993). Reduced abundance of Ixodes scapularis (Acari: Ixodidae) and Lyme disease risk by deer exclusion. J Med Entomol 30, 1043-1049.

Dattwyler, R. J., Luft, B. J., Kunkel, M. J., Finkel, M. F., Wormser, G. P., Rush, T. J. & other authors (1997). Ceftriaxone compared with doxycycline for the treatment of acute disseminated Lyme disease. N Engl J Med 337, 289-294.

de Silva, A. M., Zeidner, N. S., Zhang, Y., Dolan, M. C., Piesman, J. & Fikrig, E. (1999). Influence of outer surface protein A antibody on Borrelia burgdorferi within feeding ticks. Infection and Immunity 67, 30-35.

Dorward, D. W., Garon, C. F. & Judd, R. C. (1989). Export and intercellular transfer of DNA via membrane blebs of Neisseria gonorrhoeae. J Bacteriol 171, 2499-2505.

Dorward, D. W., Fischer, E. R. & Brooks, D. M. (1997). Invasion and cytopathic killing of human lymphocytes by spirochetes causing Lyme disease. Clin Infect Dis 25 Suppl 1, S2-8.

Dsouli, N., Younsi-Kabachii, H., Postic, D., Nouira, S., Gern, L. & Bouattour, A. (2006). Reservoir role of lizard Psammodromus algirus in transmission cycle of Borrelia burgdorferi sensu lato (Spirochaetaceae) in Tunisia. J Med Entomol 43, 737-742.

Duffy, D. C. & Campbell, S. R. (1994). Ambient Air-Temperature as a Predictor of Activity of Adult Ixodes-Scapularis (Acari, Ixodidae). Journal of Medical Entomology 31, 178-180.

Eicken, C., Sharma, V., Klabunde, T., Owens, R. T., Pikas, D. S., Hook, M. & other authors (2001). Crystal structure of Lyme disease antigen outer surface protein C from Borrelia burgdorferi. J Biol Chem 276, 10010-10015.

Eiffert, H., Karsten, A., Thomssen, R. & Christen, H. J. (1998). Characterization of Borrelia burgdorferi strains in Lyme arthritis. Scand J Infect Dis 30, 265-268.

Ekerfelt, C., Ernerudh, J., Bunikis, J., Vrethem, M., Aagesen, J., Roberg, M. & other authors (1997). Compartmentalization of antigen specific cytokine responses to the central nervous system in CNS borreliosis: secretion of IFN-gamma predominates over IL-4 secretion in response to outer surface proteins of Lyme disease Borrelia spirochetes. J Neuroimmunol 79, 155-162.

Ekerfelt, C., Masreliez, C., Svenvik, M., Ernerudh, J., Roberg, M. & Forsberg, P. (2001). Antibodies and T-cell reactivity to Borrelia burgdorferi in an asymptomatic population: a study of healthy blood donors in an inland town district in the south-east of Sweden. Scand J Infect Dis 33, 806-808.

Ekerfelt, C., Ernerudh, J., Forsberg, P., Jonsson, A. L., Vrethem, M., Arlehag, L. & other authors (2004). Lyme borreliosis in Sweden--diagnostic performance of five commercial Borrelia serology kits using sera from well-defined patient groups. APMIS 112, 74-78.

Fahrer, H., van der Linden, S. M., Sauvain, M. J., Gern, L., Zhioua, E. & Aeschlimann, A. (1991a). The prevalence and incidence of clinical and asymptomatic Lyme borreliosis in a population at risk. J Infect Dis 163, 305-310.

Fahrer, H., Vanderlinden, S. M., Sauvain, M. J., Gern, L., Zhioua, E. & Aeschlimann, A. (1991b). The Prevalence and Incidence of Clinical and Asymptomatic Lyme Borreliosis in a Population at Risk. Journal of Infectious Diseases 163, 305-310.

Falco, R. C., Fish, D. & Piesman, J. (1996). Duration of tick bites in a Lyme disease-endemic area. Am J Epidemiol 143, 187-192.

Fingerle, V., Hauser, U., Liegl, G., Petko, B., Preac-Mursic, V. & Wilske, B. (1995). Expression of outer surface proteins A and C of Borrelia burgdorferi in Ixodes ricinus. J Clin Microbiol 33, 1867-1869.

Fischer, J. R., Parveen, N., Magoun, L. & Leong, J. M. (2003). Decorin-binding proteins A and B confer distinct mammalian cell type-specific attachment by Borrelia burgdorferi, the Lyme disease spirochete. Proc Natl Acad Sci U S A 100, 7307-7312.

Floris, R., Altobelli, A., Boemo, B., Mignozzi, K. & Cinco, M. (2006). First detection of TBE virus sequences in Ixodes ricinus from Friuli Venezia Giulia (Italy). New Microbiol 29, 147-150.

Foldvari, G., Farkas, R. & Lakos, A. (2005). Borrelia spielmanii erythema migrans, Hungary. Emerg Infect Dis 11, 1794-1795.

Forsberg, P., Ernerudh, J., Ekerfelt, C., Roberg, M., Vrethem, M. & Bergstrom, S. (1995). The outer surface proteins of Lyme disease borrelia spirochetes stimulate T cells to secrete interferon-gamma (IFN-gamma): diagnostic and pathogenic implications. Clin Exp Immunol 101, 453-460.

Fraenkel, C. J., Garpmo, U. & Berglund, J. (2002). Determination of novel Borrelia genospecies in Swedish Ixodes ricinus ticks. J Clin Microbiol 40, 3308-3312.

Fraser, C. M., Casjens, S., Huang, W. M., Sutton, G. G., Clayton, R., Lathigra, R. & other authors (1997). Genomic sequence of a Lyme disease spirochaete, Borrelia burgdorferi. Nature 390, 580-586.

Fritzsche, M. (2005). Chronic Lyme borreliosis at the root of multiple sclerosis - is a cure with antibiotics attainable? Medical Hypotheses 64, 438-448.

Fukunaga, M. & Koreki, Y. (1995). The flagellin gene of Borrelia miyamotoi sp. nov. and its phylogenetic relationship among Borrelia species. Fems Microbiol Lett 134, 255-258.

Fulop, B. & Poggensee, G. (2008). Epidemiological situation of Lyme borreliosis in Germany: surveillance data from six Eastern German States, 2002 to 2006. Parasitol Res 103 Suppl 1, S117-120.

Garg, R., Juncadella, I. J., Ramamoorthi, N., Ashish, Ananthanarayanan, S. K., Thomas, V. & other authors (2006). Cutting edge: CD4 is the receptor for the tick saliva immunosuppressor, Salp15. Journal of Immunology 177, 6579-6583.

Garin, C. & Bujadoux, R. (1922). Paralysie par les Tiques. J Med Lyon, 765-767.

Garon, C. F., Dorward, D. W. & Corwin, M. D. (1989). Structural features of Borrelia burgdorferi--the Lyme disease spirochete: silver staining for nucleic acids. Scanning Microsc Suppl 3, 109-115.

Gern, L. (2008). Borrelia burgdorferi sensu lato, the agent of lyme borreliosis: life in the wilds. Parasite 15, 244-247.

Girschick, H. J., Huppertz, H. I., Russmann, H., Krenn, V. & Karch, H. (1996). Intracellular persistence of Borrelia burgdorferi in human synovial cells. Rheumatol Int 16, 125-132.

Grab, D. J., Lanners, H., Martin, L. N., Chesney, J., Cai, C., Adkisson, H. D. & other authors (1999). Interaction of Borrelia burgdorferi with peripheral blood fibrocytes, antigen-presenting cells with the potential for connective tissue targeting. Mol Med 5, 46-54.

Grab, D. J., Perides, G., Dumler, J. S., Kim, K. J., Park, J., Kim, Y. V. & other authors (2005). Borrelia burgdorferi, host-derived proteases, and the blood-brain barrier. Infect Immun 73, 1014-1022.

Gray, J., Stanek, G., Kundi, M. & Kocianova, E. (2005). Dimensions of engorging Ixodes ricinus as a measure of feeding duration. Int J Med Microbiol 295, 567-572.

Gray, J. S. (1991). The development and seasonal activity of the tick Ixodes ricinus: a vector of Lyme Borreliosis. Rev Med Vet Entomol 79, 232-333.

Gray, J. S. (1998). The ecology of ticks transmitting Lyme borreliosis. Experimental & Applied Acarology 22, 249-258.

Gugliotta, J. L., Goethert, H. K., Berardi, V. P. & Telford III, S. R. (2013). Meningoencephalitis from Borrelia miyamotoi in an Immunocompromised Patient. N Engl J Med 368, 240-245.

Gurycova, D., Kocianova, E., Vyrostekova, V. & Rehacek, J. (1995). Prevalence of ticks infected with Francisella tularensis in natural foci of tularemia in western Slovakia. Eur J Epidemiol 11, 469-474.

Gustafson, R., Forsgren, M., Gardulf, A., Granstrom, M. & Svenungsson, B. (1993a). Clinical manifestations and antibody prevalence of Lyme borreliosis and tick-borne encephalitis in Sweden: a study in five endemic areas close to Stockholm. Scand J Infect Dis 25, 595-603.

Gustafson, R., Forsgren, M., Gardulf, A., Granstrom, M. & Svenungsson, B. (1993b). Antibody prevalence and clinical manifestations of Lyme borreliosis and tick-borne encephalitis in Swedish orienteers. Scand J Infect Dis 25, 605-611.

Halperin, J. J. (2011). Neurologic manifestations of lyme disease. Curr Infect Dis Rep 13, 360-366.

Hanincova, K., Schafer, S. M., Etti, S., Sewell, H. S., Taragelova, V., Ziak, D. & other authors (2003a). Association of Borrelia afzelii with rodents in Europe. Parasitology 126, 11-20.

Hanincova, K., Taragelova, V., Koci, J., Schafer, S. M., Hails, R., Ullmann, A. J. & other authors (2003b). Association of Borrelia garinii and B. valaisiana with songbirds in Slovakia. Appl Environ Microbiol 69, 2825-2830.

Hauser, U., Lehnert, G., Lobentanzer, R. & Wilske, B. (1997). Interpretation criteria for standardized western blots for three European species of Borrelia burgdorferi sensu lato. Journal of Clinical Microbiology 35, 1433-1444.

Hechemy, K. E., Samsonoff, W. A., Harris, H. L. & McKee, M. (1992). Adherence and entry of Borrelia burgdorferi in Vero cells. J Med Microbiol 36, 229-238.

Hellerström, S. (1951). Erythema chronicum migrans Afzelius with meningitis. Acta Derm Venereol 31, 227-234.

Henningsson, A. J., Malmvall, B. E., Ernerudh, J., Matussek, A. & Forsberg, P. (2010). Neuroborreliosis--an epidemiological, clinical and healthcare cost study from an endemic area in the south-east of Sweden. Clin Microbiol Infect 16, 1245-1251.

Herxheimer, K. & Hartman, K. (1902). Über acrodermatitis chronica atrophicans. 1902.

Hjetland, R., Eliassen, K. E., Lindbaek, M., Nilsen, R. M., Grude, N. & Ulvestad, E. (2013). Tick bites in healthy adults from western Norway: occurrence, risk factors, and outcomes. Ticks Tick Borne Dis 4, 304-310.

Hollström, E. (1951). Successful treatment of erythema migrans Afzelius. Acta Derm Venereol 31, 235-243.

Hovius, J. W., de Wever, B., Sohne, M., Brouwer, M. C., Coumou, J., Wagemakers, A. & other authors (2013). A case of meningoencephalitis by the relapsing fever spirochaete Borrelia miyamotoi in Europe. Lancet 382, 658.

Hristea, A., Hristescu, S., Ciufecu, C. & Vasile, A. (2001). Seroprevalence of Borrelia burgdorferi in Romania. Eur J Epidemiol 17, 891-896.

Hu, L. T., Perides, G., Noring, R. & Klempner, M. S. (1995). Binding of human plasminogen to Borrelia burgdorferi. Infect Immun 63, 3491-3496.

Hubalek, Z. (2009). Epidemiology of lyme borreliosis. Curr Probl Dermatol 37, 31-50.

Hugli, D., Moret, J., Rais, O., Moosmann, Y., Erard, P., Malinverni, R. & other authors (2009). Tick bites in a Lyme borreliosis highly endemic area in Switzerland. Int J Med Microbiol 299, 155-160.

Hulinska, D., Votypka, J., Vanousova, D., Hercogova, J., Hulinsky, V., Drevova, H. & other authors (2009). Identification of Anaplasma phagocytophilum and Borrelia burgdorferi sensu lato in patients with erythema migrans. Folia Microbiol (Praha) 54, 246-256.

Huppertz, H. I., Bohme, M., Standaert, S. M., Karch, H. & Plotkin, S. A. (1999). Incidence of Lyme borreliosis in the Wurzburg region of Germany. Eur J Clin Microbiol Infect Dis 18, 697-703.

Jaenson, T. G., Eisen, L., Comstedt, P., Mejlon, H. A., Lindgren, E., Bergstrom, S. & other authors (2009). Risk indicators for the tick Ixodes ricinus and Borrelia burgdorferi sensu lato in Sweden. Med Vet Entomol 23, 226-237.

Jaenson, T. G. & Lindgren, E. (2011). The range of Ixodes ricinus and the risk of contracting Lyme borreliosis will increase northwards when the vegetation period becomes longer. Ticks Tick Borne Dis 2, 44-49.

Jaenson, T. G., Jaenson, D. G., Eisen, L., Petersson, E. & Lindgren, E. (2012). Changes in the geographical distribution and abundance of the tick Ixodes ricinus during the past 30 years in Sweden. Parasit Vectors 5, 8.

Jaenson, T. G. T. & Jensen, J.-K. (2007). Records of ticks (Acari, Ixodidae) from the Faroe Islands. Norw J Entomol 54, 11-15.

Jaulhac, B., Heller, R., Limbach, F. X., Hansmann, Y., Lipsker, D., Monteil, H. & other authors (2000). Direct molecular typing of Borrelia burgdorferi sensu lato species in synovial samples from patients with lyme arthritis. J Clin Microbiol 38, 1895-1900.

Jenkins, A., Hvidsten, D., Matussek, A., Lindgren, P. E., Stuen, S. & Kristiansen, B. E. (2012). Borrelia burgdorferi sensu lato in Ixodes ricinus ticks from Norway: evaluation of a PCR test targeting the chromosomal flaB gene. Exp Appl Acarol 58, 431-439.

Jensen, P. M., Hansen, H. & Frandsen, F. (2000). Spatial risk assessment for Lyme borreliosis in Denmark. Scand J Infect Dis 32, 545-550.

Johnson, R. C., Schmid, G. P., Hyde, F. W., Steigerwalt, A. G. & Brenner, D. J. (1984). Borrelia-Burgdorferi Sp-Nov - Etiologic Agent of Lyme-Disease. Int J Syst Bacteriol 34, 496-497.

Kahl, O., Janetzki-Mittmann, C., Gray, J. S., Jonas, R., Stein, J. & de Boer, R. (1998). Risk of infection with Borrelia burgdorferi sensu lato for a host in relation to the duration of nymphal Ixodes ricinus feeding and the method of tick removal. Zentralbl Bakteriol 287, 41-52.

Keller, A., Graefen, A., Ball, M., Matzas, M., Boisguerin, V., Maixner, F. & other authors (2012). New insights into the Tyrolean Iceman's origin and phenotype as inferred by whole-genome sequencing. Nat Commun 3, 698.

Kenedy, M. R., Lenhart, T. R. & Akins, D. R. (2012). The role of Borrelia burgdorferi outer surface proteins. FEMS Immunol Med Microbiol 66, 1-19.

Kiffner, C., Lodige, C., Alings, M., Vor, T. & Ruhe, F. (2011). Attachment site selection of ticks on roe deer, Capreolus capreolus. Exp Appl Acarol 53, 79-94.

Kiszewski, A. E., Matuschka, F. R. & Spielman, A. (2001). Mating strategies and spermiogenesis in ixodid ticks. Annu Rev Entomol 46, 167-182.

Klempner, M. S., Noring, R. & Rogers, R. A. (1993). Invasion of human skin fibroblasts by the Lyme disease spirochete, Borrelia burgdorferi. J Infect Dis 167, 1074-1081.

Klempner, M. S., Noring, R., Epstein, M. P., McCloud, B., Hu, R., Limentani, S. A. & other authors (1995). Binding of human plasminogen and urokinase-type plasminogen activator to the Lyme disease spirochete, Borrelia burgdorferi. J Infect Dis 171, 1258-1265.

Klompen, H. & Grimaldi, D. (2001). First mesozoic record of a parasitiform mite: a larval argasid tick in cretaceous amber (acari: ixodida: argasidae). Ann Entomol Soc Am 94, 10-15.

Korenberg, E. I., Gorelova, N. B. & Kovalevskii, Y. V. (2002). Ecology of Borrelia burgdorferi sensu lato in Russia. In *Lyme Borreliosis: Biology, Epidemiology and Control*, pp. 175-200. Edited by J. Gray, O. Kahl & G. Stanek. New York: CAB International.

Krause, P. J., Narasimhan, S., Wormser, G. P., Rollend, L., Fikrig, E., Lepore, T. & other authors (2013). Human Borrelia miyamotoi infection in the United States. N Engl J Med 368, 291-293.

Kurtenbach, K., Sewell, H. S., Ogden, N. H., Randolph, S. E. & Nuttall, P. A. (1998). Serum complement sensitivity as a key factor in Lyme disease ecology. Infect Immun 66, 1248-1251.

Kurtenbach, K., De Michelis, S., Etti, S., Schafer, S. M., Sewell, H. S., Brade, V. & other authors (2002a). Host association of Borrelia burgdorferi sensu lato--the key role of host complement. Trends Microbiol 10, 74-79.

Kurtenbach, K., De Michelis, S., Sewell, H. S., Etti, S., Schafer, S. M., Holmes, E. & other authors (2002b). The key roles of selection and migration in the ecology of Lyme borreliosis. Int J Med Microbiol 291 Suppl 33, 152-154.

Lathrop, S. L., Ball, R., Haber, P., Mootrey, G. T., Braun, M. M., Shadomy, S. V. & other authors (2002). Adverse event reports following vaccination for Lyme disease: December 1998-July 2000. Vaccine 20, 1603-1608.

Lebech, A. M., Hansen, K., Brandrup, F., Clemmensen, O. & Halkier-Sorensen, L. (2000). Diagnostic value of PCR for detection of Borrelia burgdorferi DNA in clinical specimens from patients with erythema migrans and Lyme neuroborreliosis. Mol Diagn 5, 139-150.

LeFebvre, R. B. & Perng, G. C. (1989). Genetic and antigenic characterization of Borrelia coriaceae, putative agent of epizootic bovine abortion. J Clin Microbiol 27, 389-393.

Lennhoff, C. (1948). Spirochaetes in aetiologically obscure diseases. Acta Derm Venereol 1948, 294-324.

Lenormand, C., Jaulhac, B., De Martino, S., Barthel, C. & Lipsker, D. (2009). Species of Borrelia burgdorferi complex that cause borrelial lymphocytoma in France. Brit J Dermatol 161, 174-176.

Leong, J. M., Wang, H., Magoun, L., Field, J. A., Morrissey, P. E., Robbins, D. & other authors (1998). Different classes of proteoglycans contribute to the attachment of Borrelia burgdorferi to cultured endothelial and brain cells. Infect Immun 66, 994-999.

Li, C., Motaleb, A., Sal, M., Goldstein, S. F. & Charon, N. W. (2000). Spirochete periplasmic flagella and motility. J Mol Microbiol Biotechnol 2, 345-354.

Liang, F. T., Nowling, J. M. & Philipp, M. T. (2000). Cryptic and exposed invariable regions of VlsE, the variable surface antigen of Borrelia burgdorferi sl. J Bacteriol 182, 3597-3601.

Liang, F. T., Yan, J., Mbow, M. L., Sviat, S. L., Gilmore, R. D., Mamula, M. & other authors (2004). Borrelia burgdorferi changes its surface antigenic expression in response to host immune responses. Infect Immun 72, 5759-5767.

Limbach, F. X., Jaulhac, B., Puechal, X., Monteil, H., Kuntz, J. L., Piemont, Y. & other authors (2001). Treatment resistant Lyme arthritis caused by Borrelia garinii. Ann Rheum Dis 60, 284-286.

Lindgren, E., Talleklint, L. & Polfeldt, T. (2000). Impact of climatic change on the northern latitude limit and population density of the disease-transmitting European tick Ixodes ricinus. Environ Health Perspect 108, 119-123.

Livengood, J. A. & Gilmore, R. D., Jr. (2006). Invasion of human neuronal and glial cells by an infectious strain of Borrelia burgdorferi. Microbes Infect 8, 2832-2840.

Lowe, B., Avila, H. A., Bloom, F. R., Gleeson, M. & Kusser, W. (2003). Quantitation of gene expression in neural precursors by reverse-transcription polymerase chain reaction using self-quenched, fluorogenic primers. Analytical Biochemistry 315, 95-105.

Luft, B. J., Jiang, W., Munoz, P., Dattwyler, R. J. & Gorevic, P. D. (1989). Biochemical and immunological characterization of the surface proteins of Borrelia burgdorferi. Infect Immun 57, 3637-3645.

116

Luft, B. J., Dattwyler, R. J., Johnson, R. C., Luger, S. W., Bosler, E. M., Rahn, D. W. & other authors (1996). Azithromycin compared with amoxicillin in the treatment of erythema migrans. A double-blind, randomized, controlled trial. Ann Intern Med 124, 785-791.

Luger, S. W., Paparone, P., Wormser, G. P., Nadelman, R. B., Grunwaldt, E., Gomez, G. & other authors (1995). Comparison of cefuroxime axetil and doxycycline in treatment of patients with early Lyme disease associated with erythema migrans. Antimicrob Agents Chemother 39, 661-667.

Läkemedelsverket (2009).Läkemedelsbehandling av borreliainfektion. Report no. 4:2009. Retrieved from http://www.lakemedelsverket.se/. 2014-03-14.

Ma, Y., Sturrock, A. & Weis, J. J. (1991). Intracellular localization of Borrelia burgdorferi within human endothelial cells. Infect Immun 59, 671-678.

Magnarelli, L. A., Norris, S. J. & Fikrig, E. (2012). Serum antibodies to whole-cell and recombinant antigens of Borrelia burgdorferi in cottontail rabbits. J Wildl Dis 48, 12-20.

Maraspin, V., Ruzic-Sabljic, E., Cimperman, J., Lotric-Furlan, S., Jurca, T., Picken, R. N. & other authors (2001). Isolation of Borrelia burgdorferi sensu lato from blood of patients with erythema migrans. Infection 29, 65-70.

Maraspin, V., Cimperman, J., Lotric-Furlan, S., Ruzic-Sabljic, E., Jurca, T., Picken, R. N. & other authors (2002). Solitary borrelial lymphocytoma in adult patients. Wien Klin Wochenschr 114, 515-523.

Masuzawa, T., Komikado, T., Iwaki, A., Suzuki, H., Kaneda, K. & Yanagihara, Y. (1996). Characterization of Borrelia sp. isolated from Ixodes tanuki, I. turdus, and I. columnae in Japan by restriction fragment length polymorphism of rrf (5S)-rrl (23S) intergenic spacer amplicons. Fems Microbiol Lett 142, 77-83.

McNeil, E., Hinshaw, W. R. & Kissling, R. E. (1949). A Study of Borrelia Anserina Infection (Spirochetosis) in Turkeys. J Bacteriol 57, 191-206.

Mehnert, W. H. & Krause, G. (2005). Surveillance of Lyme borreliosis in Germany, 2002 and 2003. Euro Surveill 10, 83-85.

Mejlon, H. A. & Jaenson, T. G. (1993). Seasonal prevalence of Borrelia burgdorferi in Ixodes ricinus in different vegetation types in Sweden. Scand J Infect Dis 25, 449-456.

Mejlon, H. A. & Jaenson, T. G. T. (1997). Questing behaviour of Ixodes ricinus ticks (Acari: Ixodidae). Experimental & Applied Acarology 21, 747-754.

Miklossy, J., Kasas, S., Zurn, A. D., McCall, S., Yu, S. & McGeer, P. L. (2008). Persisting atypical and cystic forms of Borrelia burgdorferi and local inflammation in Lyme neuroborreliosis. J Neuroinflammation 5, 40.

Montgomery, R. R., Nathanson, M. H. & Malawista, S. E. (1993). The fate of Borrelia burgdorferi, the agent for Lyme disease, in mouse macrophages. Destruction, survival, recovery. J Immunol 150, 909-915.

Moran Cadenas, F., Rais, O., Humair, P. F., Douet, V., Moret, J. & Gern, L. (2007). Identification of host bloodmeal source and Borrelia burgdorferi sensu lato in field-collected Ixodes ricinus ticks in Chaumont (Switzerland). J Med Entomol 44, 1109-1117.

Mygland, A., Ljostad, U., Fingerle, V., Rupprecht, T., Schmutzhard, E. & Steiner, I. (2010). EFNS guidelines on the diagnosis and management of European Lyme neuroborreliosis. Eur J Neurol 17, 8-16, e11-14.

Nadelman, R. B., Luger, S. W., Frank, E., Wisniewski, M., Collins, J. J. & Wormser, G. P. (1992). Comparison of cefuroxime axetil and doxycycline in the treatment of early Lyme disease. Ann Intern Med 117, 273-280.

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Nadelman, R. B. & Wormser, G. P. (1998). Lyme borreliosis. Lancet 352, 557-565.

Nava, S., Guglielmone, A. A. & Mangold, A. J. (2009). An overview of systematics and evolution of ticks. Front Biosci 14, 2857-2877.

Nigrovic, L. E. & Thompson, K. M. (2007). The Lyme vaccine: a cautionary tale. Epidemiol Infect 135, 1-8.

Nowakowski, J., Nadelman, R. B., Sell, R., McKenna, D., Cavaliere, L. F., Holmgren, D. & other authors (2003). Long-term follow-up of patients with culture-confirmed Lyme disease. Am J Med 115, 91-96.

Nuttall, P. A. & Labuda, M. (2004). Tick-host interactions: saliva-activated transmission. Parasitology 129 Suppl, S177-189.

O'Rourke, M., Traweger, A., Lusa, L., Stupica, D., Maraspin, V., Barrett, P. N. & other authors (2013). Quantitative detection of Borrelia burgdorferi sensu lato in erythema migrans skin lesions using internally controlled duplex real time PCR. Plos One 8, e63968.

Ogden, N. H., Hailes, R. S. & Nuttall, P. A. (1998). Interstadial variation in the attachment sites of Ixodes ricinus ticks on sheep. Exp Appl Acarol 22, 227-232.

Ohlenbusch, A., Matuschka, F. R., Richter, D., Christen, H. J., Thomssen, R., Spielman, A. & other authors (1996). Etiology of the acrodermatitis chronica atrophicans lesion in Lyme disease. J Infect Dis 174, 421-423.

Ohnishi, J., Piesman, J. & de Silva, A. M. (2001). Antigenic and genetic heterogeneity of Borrelia burgdorferi populations transmitted by ticks. Proc Natl Acad Sci U S A 98, 670-675.

Olsen, B., Jaenson, T. G. & Bergstrom, S. (1995). Prevalence of Borrelia burgdorferi sensu lato-infected ticks on migrating birds. Appl Environ Microbiol 61, 3082-3087.

Olsen, N. J. & Kovacs, W. J. (1996). Gonadal steroids and immunity. Endocr Rev 17, 369-384.

Ornstein, K., Berglund, J., Nilsson, I., Norrby, R. & Bergstrom, S. (2001). Characterization of Lyme borreliosis isolates from patients with erythema migrans and neuroborreliosis in southern Sweden. J Clin Microbiol 39, 1294-1298.

Ornstein, K. & Barbour, A. G. (2006). A reverse transcriptase-polymerase chain reaction assay of Borrelia burgdorferi 16S rRNA for highly sensitive quantification of pathogen load in a vector. Vector Borne Zoonotic Dis 6, 103-112.

Pal, U., Li, X., Wang, T., Montgomery, R. R., Ramamoorthi, N., Desilva, A. M. & other authors (2004). TROSPA, an Ixodes scapularis receptor for Borrelia burgdorferi. Cell 119, 457-468.

Parola, P. & Raoult, D. (2001). Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. Clin Infect Dis 32, 897-928.

Paster, B. J. & Dewhirst, F. E. (2000). Phylogenetic foundation of spirochetes. J Mol Microbiol Biotechnol 2, 341-344.

Perez, D., Kneubuhler, Y., Rais, O. & Gern, L. (2012). Seasonality of Ixodes ricinus ticks on vegetation and on rodents and Borrelia burgdorferi sensu lato genospecies diversity in two lyme borreliosis-endemic areas in Switzerland. Vector Borne Zoonotic Dis 12, 633-644.

Persing, D. H., Rutledge, B. J., Rys, P. N., Podzorski, D. S., Mitchell, P. D., Reed, K. D. & other authors (1994). Target imbalance: disparity of Borrelia burgdorferi genetic material in synovial fluid from Lyme arthritis patients. J Infect Dis 169, 668-672.

Peters, D. J. & Benach, J. L. (1997). Borrelia burgdorferi adherence and injury to undifferentiated and differentiated neural cells in vitro. J Infect Dis 176, 470-477.

Picken, R. N., Strle, F., Ruzic-Sabljic, E., Maraspin, V., Lotric-Furlan, S., Cimperman, J. & other authors (1997). Molecular subtyping of Borrelia burgdorferi sensu lato isolates from five patients with solitary lymphocytoma. J Invest Dermatol 108, 92-97.

Piesman, J., Mather, T. N., Sinsky, R. J. & Spielman, A. (1987). Duration of tick attachment and Borrelia burgdorferi transmission. J Clin Microbiol 25, 557-558.

Piesman, J., Oliver, J. R. & Sinsky, R. J. (1990). Growth kinetics of the Lyme disease spirochete (Borrelia burgdorferi) in vector ticks (Ixodes dammini). Am J Trop Med Hyg 42, 352-357.

Piesman, J., Schneider, B. S. & Zeidner, N. S. (2001). Use of quantitative PCR to measure density of Borrelia burgdorferi in the midgut and salivary glands of feeding tick vectors. J Clin Microbiol 39, 4145-4148.

Platonov, A. E., Karan, L. S., Kolyasnikova, N. M., Makhneva, N. A., Toporkova, M. G., Maleev, V. V. & other authors (2011). Humans infected with relapsing fever spirochete Borrelia miyamotoi, Russia. Emerg Infect Dis 17, 1816-1823.

Postic, D., Assous, M. V., Grimont, P. A. & Baranton, G. (1994). Diversity of Borrelia burgdorferi sensu lato evidenced by restriction fragment length polymorphism of rrf (5S)-rrl (23S) intergenic spacer amplicons. Int J Syst Bacteriol 44, 743-752.

Preac-Mursic, V., Pfister, H. W., Spiegel, H., Burk, R., Wilske, B., Reinhardt, S. & other authors (1993). First isolation of Borrelia burgdorferi from an iris biopsy. J Clin Neuroophthalmol 13, 155-161; discussion 162.

Pretorius, A. M., Jensenius, M., Clarke, F. & Ringertz, S. H. (2003). Repellent efficacy of DEET and KBR 3023 against Amblyomma hebraeum (Acari: Ixodidae). J Med Entomol 40, 245-248.

Ramamoorthi, N., Narasimhan, S., Pal, U., Bao, F., Yang, X. F., Fish, D. & other authors (2005). The Lyme disease agent exploits a tick protein to infect the mammalian host. Nature 436, 573-577.

Raoult, D. & Roux, V. (1997). Rickettsioses as paradigms of new or emerging infectious diseases. Clin Microbiol Rev 10, 694-&.

Ras, N. M., Lascola, B., Postic, D., Cutler, S. J., Rodhain, F., Baranton, G. & other authors (1996). Phylogenesis of relapsing fever Borrelia spp. Int J Syst Bacteriol 46, 859-865.

Rauter, C. & Hartung, T. (2005). Prevalence of Borrelia burgdorferi sensu lato genospecies in Ixodes ricinus ticks in Europe: a metaanalysis. Appl Environ Microbiol 71, 7203-7216.

Rebaudet, S. & Parola, P. (2006). Epidemiology of relapsing fever borreliosis in Europe. FEMS Immunol Med Microbiol 48, 11-15.

Richter, D., Allgower, R. & Matuschka, F. R. (2002). Co-feeding transmission and its contribution to the perpetuation of the lyme disease spirochete Borrelia afzelii. Emerg Infect Dis 8, 1421-1425.

Richter, D., Schlee, D. B., Allgower, R. & Matuschka, F. R. (2004). Relationships of a novel Lyme disease spirochete, Borrelia spielmani sp. nov., with its hosts in Central Europe. Appl Environ Microbiol 70, 6414-6419.

Richter, D. & Matuschka, F. R. (2006). Perpetuation of the Lyme disease spirochete Borrelia lusitaniae by lizards. Appl Environ Microbiol 72, 4627-4632.

Richter, D., Debski, A., Hubalek, Z. & Matuschka, F. R. (2012). Absence of Lyme disease spirochetes in larval Ixodes ricinus ticks. Vector Borne Zoonotic Dis 12, 21-27.

Robertson, J., Guy, E., Andrews, N., Wilske, B., Anda, P., Granstrom, M. & other authors (2000). A European multicenter study of immunoblotting in serodiagnosis of lyme borreliosis. J Clin Microbiol 38, 2097-2102.

Rodgers, S. E., Zolnik, C. P. & Mather, T. N. (2007). Duration of exposure to suboptimal atmospheric moisture affects nymphal blacklegged tick survival. Journal of Medical Entomology 44, 372-375.

Rollend, L., Fish, D. & Childs, J. E. (2013). Transovarial transmission of Borrelia spirochetes by Ixodes scapularis: A summary of the literature and recent observations. Ticks Tick Borne Dis 4, 46-51.

Rosa, P. A., Tilly, K. & Stewart, P. E. (2005). The burgeoning molecular genetics of the Lyme disease spirochaete. Nature Reviews Microbiology 3, 129-143.

Rupprecht, T. A., Koedel, U., Heimerl, C., Fingerle, V., Paul, R., Wilske, B. & other authors (2006). Adhesion of Borrelia garinii to neuronal cells is mediated by the interaction of OspA with proteoglycans. J Neuroimmunol 175, 5-11.

Rupprecht, T. A., Koedel, U., Fingerle, V. & Pfister, H. W. (2008). The pathogenesis of lyme neuroborreliosis: from infection to inflammation. Mol Med 14, 205-212.

Ruzic-Sabljic, E., Strle, F., Cimperman, J., Maraspin, V., Lotric-Furlan, S. & Pleterski-Rigler, D. (2000). Characterisation of Borrelia burgdorferi sensu lato strains isolated from patients with skin manifestations of Lyme borreliosis residing in Slovenia. J Med Microbiol 49, 47-53.

Sambri, V., Basso, F., Massaria, F., Ardizzoni, M. & Cevenini, R. (1993). Adherence of Borrelia burgdorferi and Borrelia hermsii to mammalian cells in vitro. New Microbiol 16, 43-49.

SBU (2013). Behandlingstid vid borreliainfektion, pp. SBU Alert-rapport nr 2013-2005: ISSN 1652-7151. Stockholm: Statens beredning för medicinsk utvärdering.

Schmidt, B. L. (1997). PCR in laboratory diagnosis of human Borrelia burgdorferi infections. Clin Microbiol Rev 10, 185-201.

Schmidtmann, E. T., Carroll, J. F. & Watson, D. W. (1998). Attachment-site patterns of adult blacklegged ticks (Acari: Ixodidae) on white-tailed deer and horses. J Med Entomol 35, 59-63.

Schwan, T. G., Piesman, J., Golde, W. T., Dolan, M. C. & Rosa, P. A. (1995). Induction of an outer surface protein on Borrelia burgdorferi during tick feeding. Proc Natl Acad Sci U S A 92, 2909-2913.

Scoles, G. A., Papero, M., Beati, L. & Fish, D. (2001). A relapsing fever group spirochete transmitted by Ixodes scapularis ticks. Vector Borne Zoonotic Dis 1, 21-34.

Scrimenti, R. (1970). Erythema chronicum migrans. Arch Dermatol 102, 104-105.

Shih, C. M., Pollack, R. J., Telford, S. R., 3rd & Spielman, A. (1992). Delayed dissemination of Lyme disease spirochetes from the site of deposition in the skin of mice. J Infect Dis 166, 827-831.

Sjowall, J., Carlsson, A., Vaarala, O., Bergstrom, S., Ernerudh, J., Forsberg, P. & other authors (2005). Innate immune responses in Lyme borreliosis: enhanced tumour necrosis factor-alpha and interleukin-12 in asymptomatic individuals in response to live spirochetes. Clin Exp Immunol 141, 89-98.

Smith, R. P., Schoen, R. T., Rahn, D. W., Sikand, V. K., Nowakowski, J., Parenti, D. L. & other authors (2002). Clinical characteristics and treatment outcome of early Lyme disease in patients with microbiologically confirmed erythema migrans. Ann Intern Med 136, 421-428.

Stanek, G., Flamm, H., Groh, V., Hirschl, A., Kristoferitsch, W., Neumann, R. & other authors (1987). Epidemiology of borrelia infections in Austria. Zentralbl Bakteriol Mikrobiol Hyg A 263, 442-449.

Stanek, G., Klein, J., Bittner, R. & Glogar, D. (1990). Isolation of Borrelia burgdorferi from the myocardium of a patient with longstanding cardiomyopathy. N Engl J Med 322, 249-252.

Stanek, G., O'Connell, S., Cimmino, M., Aberer, E., Kristoferitsch, W., Granstrom, M. & other authors (1996). European Union Concerted Action on Risk Assessment in Lyme Borreliosis: clinical case definitions for Lyme borreliosis. Wien Klin Wochenschr 108, 741-747.

Stanek, G. & Strle, F. (2003). Lyme borreliosis. Lancet 362, 1639-1647.

Stanek, G., Fingerle, V., Hunfeld, K. P., Jaulhac, B., Kaiser, R., Krause, A. & other authors (2011). Lyme borreliosis: clinical case definitions for diagnosis and management in Europe. Clin Microbiol Infect 17, 69-79.

Stanek, G. & Reiter, M. (2011). The expanding Lyme Borrelia complex--clinical significance of genomic species? Clin Microbiol Infect 17, 487-493.

Steere, A. C., Malawista, S. E., Snydman, D. R., Shope, R. E., Andiman, W. A., Ross, M. R. & other authors (1977). Lyme arthritis: an epidemic of oligoarticular arthritis in children and adults in three connecticut communities. Arthritis Rheum 20, 7-17.

Steere, A. C., Broderick, T. F. & Malawista, S. E. (1978). Erythema chronicum migrans and Lyme arthritis: epidemiologic evidence for a tick vector. Am J Epidemiol 108, 312-321.

Steere, A. C., Batsford, W. P., Weinberg, M., Alexander, J., Berger, H. J., Wolfson, S. & other authors (1980). Lyme carditis: cardiac abnormalities of Lyme disease. Ann Intern Med 93, 8-16.

Steere, A. C., Hutchinson, G. J., Rahn, D. W., Sigal, L. H., Craft, J. E., DeSanna, E. T. & other authors (1983). Treatment of the early manifestations of Lyme disease. Ann Intern Med 99, 22-26.

Steere, A. C. (2001). Lyme disease. N Engl J Med 345, 115-125.

Steere, A. C., Coburn, J. & Glickstein, L. (2004). The emergence of Lyme disease. J Clin Invest 113, 1093-1101.

Stevenson, B., El-Hage, N., Hines, M. A., Miller, J. C. & Babb, K. (2002). Differential binding of host complement inhibitor factor H by Borrelia burgdorferi Erp surface proteins: a possible mechanism underlying the expansive host range of Lyme disease spirochetes. Infect Immun 70, 491-497.

Stjernberg, L. & Berglund, J. (2002). Risk of acquiring tick bites in south-eastern Sweden. Scand J Infect Dis 34, 840-844.

Strle, F., Pleterski-Rigler, D., Stanek, G., Pejovnik-Pustinek, A., Ruzic, E. & Cimperman, J. (1992). Solitary borrelial lymphocytoma: report of 36 cases. Infection 20, 201-206.

Strle, F., Preac-Mursic, V., Cimperman, J., Ruzic, E., Maraspin, V. & Jereb, M. (1993). Azithromycin versus doxycycline for treatment of erythema migrans: clinical and microbiological findings. Infection 21, 83-88.

Strle, F., Nelson, J. A., Ruzic-Sabljic, E., Cimperman, J., Maraspin, V., Lotric-Furlan, S. & other authors (1996). European Lyme borreliosis: 231 culture-confirmed cases involving patients with erythema migrans. Clin Infect Dis 23, 61-65.

Strle, F. (1999). Principles of the diagnosis and antibiotic treatment of Lyme borreliosis. Wien Klin Wochenschr 111, 911-915.

Strle, F., Videcnik, J., Zorman, P., Cimperman, J., Lotric-Furlan, S. & Maraspin, V. (2002). Clinical and epidemiological findings for patients with erythema migrans. Comparison of cohorts from the years 1993 and 2000. Wien Klin Wochenschr 114, 493-497.

Strle, F. & Stanek, G. (2009). Clinical manifestations and diagnosis of lyme borreliosis. Curr Probl Dermatol 37, 51-110.

Strle, F., Wormser, G. P., Mead, P., Dhaduvai, K., Longo, M. V., Adenikinju, O. & other authors (2013). Gender disparity between cutaneous and non-cutaneous manifestations of Lyme borreliosis. Plos One 8, e64110.

Suss, J. (2003). Epidemiology and ecology of TBE relevant to the production of effective vaccines. Vaccine 21 Suppl 1, S19-35.

Swanson, S. J., Neitzel, D., Reed, K. D. & Belongia, E. A. (2006). Coinfections acquired from ixodes ticks. Clin Microbiol Rev 19, 708-727.

Szer, I. S., Taylor, E. & Steere, A. C. (1991). The long-term course of Lyme arthritis in children. N Engl J Med 325, 159-163.

Takayama, K., Rothenberg, R. J. & Barbour, A. G. (1987). Absence of lipopolysaccharide in the Lyme disease spirochete, Borrelia burgdorferi. Infect Immun 55, 2311-2313.

Talleklint, L. & Jaenson, T. G. (1996). Seasonal variations in density of questing Ixodes ricinus (Acari: Ixodidae) nymphs and prevalence of infection with B. burgdorferi s.l. in south central Sweden. J Med Entomol 33, 592-597.

Talleklint, L. & Jaenson, T. G. (1997). Infestation of mammals by Ixodes ricinus ticks (Acari: Ixodidae) in south-central Sweden. Exp Appl Acarol 21, 755-771.

THI (2013). Finland´s National institute for health and welfare [Statistical Database]. Retrieved from http://www.thl.fi/en_US/web/en. Helsinki, Finland.

Thomas, D. D., Cadavid, D. & Barbour, A. G. (1994). Differential association of Borrelia species with cultured neural cells. J Infect Dis 169, 445-448.

Tijsse-Klasen, E., Jacobs, J. J., Swart, A., Fonville, M., Reimerink, J. H., Brandenburg, A. H. & other authors (2011). Small risk of developing symptomatic tick-borne diseases following a tick bite in The Netherlands. Parasit Vectors 4, 17.

Tjernberg, I., Kruger, G. & Eliasson, I. (2007). C6 peptide ELISA test in the serodiagnosis of Lyme borreliosis in Sweden. Eur J Clin Microbiol Infect Dis 26, 37-42.

Tomao, P., Ciceroni, L., D'Ovidio, M. C., De Rosa, M., Vonesch, N., Iavicoli, S. & other authors (2005). Prevalence and incidence of antibodies to Borrelia burgdorferi and to tick-borne encephalitis virus in agricultural and forestry workers from Tuscany, Italy. Eur J Clin Microbiol Infect Dis 24, 457-463.

Tsao, J. I., Wootton, J. T., Bunikis, J., Luna, M. G., Fish, D. & Barbour, A. G. (2004). An ecological approach to preventing human infection: vaccinating wild mouse reservoirs intervenes in the Lyme disease cycle. Proc Natl Acad Sci U S A 101, 18159-18164.

Wahlberg, P. (1990). Incidence of tick-bite in man in Aland Islands: reference to the spread of Lyme borreliosis. Scand J Infect Dis 22, 59-62.

van der Heijden, I. M., Wilbrink, B., Rijpkema, S. G., Schouls, L. M., Heymans, P. H., van Embden, J. D. & other authors (1999). Detection of Borrelia burgdorferi sensu stricto by reverse line blot in the joints of Dutch patients with Lyme arthritis. Arthritis Rheum 42, 1473-1480.

van der Linde, M. R. (1991). Lyme carditis: clinical characteristics of 105 cases. Scand J Infect Dis Suppl 77, 81-84.

Wang, G., van Dam, A. P. & Dankert, J. (1999a). Phenotypic and genetic characterization of a novel Borrelia burgdorferi sensu lato isolate from a patient with lyme borreliosis. J Clin Microbiol 37, 3025-3028.

Wang, G., van Dam, A. P., Schwartz, I. & Dankert, J. (1999b). Molecular typing of Borrelia burgdorferi sensu lato: taxonomic, epidemiological, and clinical implications. Clin Microbiol Rev 12, 633-653.

Wang, G. (2002). Direct detection methods for Lyme Borrelia, including the use of quantitative assays. Vector Borne Zoonotic Dis 2, 223-231.

Wang, G., Liveris, D., Brei, B., Wu, H., Falco, R. C., Fish, D. & other authors (2003). Real-time PCR for simultaneous detection and quantification of Borrelia burgdorferi in field-collected Ixodes scapularis ticks from the Northeastern United States. Appl Environ Microbiol 69, 4561-4565.

Wang, I. N., Dykhuizen, D. E., Qiu, W., Dunn, J. J., Bosler, E. M. & Luft, B. J. (1999c). Genetic diversity of ospC in a local population of Borrelia burgdorferi sensu stricto. Genetics 151, 15-30.

Vannier, E. & Krause, P. J. (2012). Human Babesiosis REPLY. New Engl J Med 367, 1071-1072.

Vasiliu, V., Herzer, P., Rossler, D., Lehnert, G. & Wilske, B. (1998). Heterogeneity of Borrelia burgdorferi sensu lato demonstrated by an ospA-type-specific PCR in synovial fluid from patients with Lyme arthritis. Med Microbiol Immunol 187, 97-102.

Vasudevan, B. & Chatterjee, M. (2013). Lyme borreliosis and skin. Indian J Dermatol 58, 167-174.

Welinder-Olsson, C., Kjellin, E., Vaht, K., Jacobsson, S. & Wenneras, C. (2010). First Case of Human "Candidatus Neoehrlichia mikurensis" Infection in a Febrile Patient with Chronic Lymphocytic Leukemia. Journal of Clinical Microbiology 48, 1956-1959.

Werner, M., Nordin, P., Arnholm, B., Elgefors, B. & Krantz, I. (2001). Borrelia burgdorferi antibodies in outdoor and indoor workers in south-west Sweden. Scand J Infect Dis 33, 128-131.

Wilske, B., Barbour, A. G., Bergstrom, S., Burman, N., Restrepo, B. I., Rosa, P. A. & other authors (1992). Antigenic variation and strain heterogeneity in Borrelia spp. Res Microbiol 143, 583-596.

Wilske, B. (2005). Epidemiology and diagnosis of Lyme borreliosis. Ann Med 37, 568-579.

Wilske, B., Fingerle, V. & Schulte-Spechtel, U. (2007). Microbiological and serological diagnosis of Lyme borreliosis. FEMS Immunol Med Microbiol 49, 13-21.

Wilson, M. L., Levine, J. F. & Spielman, A. (1984). Effect of deer reduction on abundance of the deer tick (Ixodes dammini). Yale J Biol Med 57, 697-705.

Woldehiwet, Z. (2010). The natural history of Anaplasma phagocytophilum. Vet Parasitol 167, 108-122.

Voordouw, M. J., Tupper, H., Onder, O., Devevey, G., Graves, C. J., Kemps, B. D. & other authors (2013). Reductions in Human Lyme Disease Risk Due to the Effects of Oral Vaccination on Tick-to-Mouse and Mouse-to-Tick Transmission. Vector-Borne Zoonot 13, 203-214.

Wormser, G. P., Nadelman, R. B., Nowakowski, J. & Schwartz, I. (2001). Asymptomatic Borrelia burgdorferi infection. Med Hypotheses 57, 435-438.

Wormser, G. P., Dattwyler, R. J., Shapiro, E. D., Halperin, J. J., Steere, A. C., Klempner, M. S. & other authors (2006). The clinical assessment, treatment, and prevention of lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America. Clin Infect Dis 43, 1089-1134.

Wressnigg, N., Pollabauer, E. M., Aichinger, G., Portsmouth, D., Low-Baselli, A., Fritsch, S. & other authors (2013). Safety and immunogenicity of a novel multivalent OspA vaccine against Lyme borreliosis in healthy adults: a double-blind, randomised, dose-escalation phase 1/2 trial. Lancet Infect Dis 13, 680-689.

Wu, J., Weening, E. H., Faske, J. B., Hook, M. & Skare, J. T. (2011). Invasion of eukaryotic cells by Borrelia burgdorferi requires beta(1) integrins and Src kinase activity. Infect Immun 79, 1338-1348.

Zhioua, E., Bouattour, A., Hu, C. M., Gharbi, M., Aeschliman, A., Ginsberg, H. S. & other authors (1999). Infection of Ixodes ricinus (Acari : Ixodidae) by Borrelia burgdorferi sensu lato in North Africa. Journal of Medical Entomology 36, 216-218.

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