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Detection and Genetic Characterization of *Borrelia* Species Circulating in Tick Population in Estonia

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.

/Julia Geller/

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Eesti puugipopulatsioonis ringlevate *Borrelia* liikide tuvastamine ja geneetiline iseloomustus

JULIA GELLER

TTÜ KIRJASTUS

To my daughter Amalia

Contents

INTRODUCTION

Over the centuries humankind has faced various emerging diseases which, with thousands or even millions of victims, had influenced the human history. In 2007, the World Health Organization (WHO) declared in its report that in the 21st century emerging diseases would be major threats of global public health. More than 60% of the emerging diseases originate in wildlife. Some pathogens can infect humans directly via aerosol, scratches, bites, food etc., while others need a vector – a flea, louse, mosquito or a tick. Those pathogens that use a tick-vector are called tick-borne pathogens (TBP). The most widely known and the most harmful tick-borne disease in the Northern hemisphere is tick-borne encephalitis virus, while Lyme borreliosis (LB), or tick-borne borreliosis, is the most common: more than 85 000 cases are registered annually in Europe. In Estonia, during the years 2002-2011 the morbidity of LB has increased seven times: from 23.4 to 171.8 cases per 100 000 population. Albeit from 2012 the infection rate of LB in humans in Estonia has decreased, it still remains one of the highest in Europe.

Lyme borreliosis is a multisystemic disease that may affect cardiovascular and neural systems, joints and eyes. It is caused by the spirochetes of a *Borrelia burgdorferi* sensu lato complex. To this day, of the 18 genospecies comprising this complex, 5 are known to be pathogenic to humans: *B. afzelii, B. garinii, B. bavariensis, B. burgdorferi* sensu stricto and *B. spielmanii*.

Another *Borrelia* genospecies that refers to the relapsing fever group spirochetes and is widely spread in Europe is *B. miyamotoi*. It has been recently published that *B. miyamotoi* causes a multisystemic disorder with viral-like symptoms and 1-2 relapses of fever.

This study was undertaken considering the high and increasing LB infection rate in Estonia. Its aim was to detect, for the first time in Estonia, *Borrelia* genospecies of *B. burgdorferi* s.l. complex as well as *B. miyamotoi* in tick populations and estimate the prevalence rates. The fact that Estonia is situated in a unique region in Europe where two main vectors of tick-borne pathogens, *Ixodes ricinus* and *I. persulcatus*, overlap, may also provide a special model for the investigation of TBPs and the relationships between the pathogen, natural hosts and different vectors. Thus, it was hypothesized that *Borrelia* in Estonia may have specific features due to two tick species distribution, as it was previously described for TBEV (1).

ORIGINAL PUBLICATIONS

The present dissertation is based on three original publications which will be referred to in the text by a corresponding Roman number:

I. **Geller J**, Nazarova L., Katargina O., Leivits A., Järvekülg L. and Golovljova I. Tick-borne pathogens in ticks feeding on migratory passerines in Western part of Estonia. Vector Borne Zoonotic Dis. 2013 Jul;13(7):443-8.

II. **Geller J.**, Nazarova L., Katargina O., Järvekülg L., Fomenko N. and Golovljova I. Detection and genetic characterization of relapsing fever spirochete *Borrelia miyamotoi* in Estonian ticks. PLoS One. 2012;7(12):e51914.

III. **Geller J.**, Nazarova L., Katargina O. and Golovljova I. *Borrelia burgdorferi* sensu lato prevalence in tick populations in Estonia. Parasit Vectors. 2013 Jul 9;6:202.

Author's contribution

I The author wrote the article and participated in conceiving and designing the experiments, performing the experiments and analyzing data.

II The author performed the experiments and participated in field collections, morphological identification procedure, conceiving and designing the experiments, analyzing data and writing the article.

III The author wrote the article and participated in field collections, morphological identification procedure, conceiving and designing the experiments, performing the experiments and analyzing data.

ABBREVIATIONS

ACA – acrodermatitis chronic atrophicans

DNA – deoxyribonucleic acid

EM – erythema migrans

glpQ – glycerophosphodiester phosphodiesterase

flaA – flagellar filament outsheath protein

flaB – flagellin protein

IGS – intergenic spacer region

LB – Lyme borreliosis

NB – neuroborreliosis

ospA – outer surface protein A

ospC – outer surface protein C

PBS – phosphate buffered saline

PCR – polymerase chain reaction

p66 – outer membrane spanning protein

RF – relapsing fever

RNA – ribonucleic acid

RT-PCR – reverse transcriptase poLymerase chain reaction

16S rRNA – small subunit of ribosomal ribonucleic acid

TBEV – tick-borne encephalitis virus

TBP – tick-borne pathogen

TROSPA – tick receptor for outer surface protein A

1. REVIEW OF THE LITERATURE

1.1. The historical overview

In 2010 National Geographic revealed the results of an autopsy and DNA analysis of a 5,300 year old mummy, known as Ötzi the Iceman (Figure 1). This man of Neolithic age became the earliest known human with Lyme infection, as in his tissues *Borrelia burgdorferi* DNA was detected (2). However, the first mentions of the symptoms known at present to be associated with Lyme disease belong to the 18th century. After visiting the island of Jura in Scotland in 1764, Reverend Dr. John Walker wrote about a "singular disease" with "exquisite pain [over] interior parts of the limbs" that started after a bite of a tick which he called a „worm" (3).

Figure 1. Ötzi the Iceman (Image source: South Tyrol Museum of Archaeology http://www.archaeologiemuseum.it/en/node/245).

At the end of the 19th century, in 1883, the German physician Alfred Buchwald described the first case of acrodermatitis chronic atrophicans (4). A research of a skin rash called erythema migrans (EM), that is also a skin manifestation of the early LB, was presented by Dr. Arvid Afzelius in 1909 at the meeting of the Swedish Society of Dermatology (5). Later, Afzelius (6) and Hellerström (7) proposed that these characteristic symptoms along with meningitic symptoms and signs might be caused by a tick bite. Penicillin was taken into use to treat erythema migrans in the 1950s after finding of spirochete-like structures in the patients' skin specimens (8-10).

Lyme disease got its name in 1976 after the town Lyme, Connecticut, USA where Dr. Allen Steere first described an unknown disease among town inhabitants (11). The typical clinical picture included an expanding red annular lesion at the site of a tick bite, arthritic symptoms, mostly in knee joints, but also neurological or cardiologic symptoms, sometimes accompanied with fatigue, fever, myalgia, chills, vomiting etc. (12-14). The arthropod tick vector was suggested for the disease because of the seasonal increase of cases during summer and early fall (11). The pathogen itself – a long irregularly coiled spirochete – was discovered by Willy Burgdorfer in 1981 while he examined the midgut of *Ixodes scapularis* (formerly *I. dammini*) tick (15). Further investigations revealed that it belonged to the *Borrelia* genus, and in 1984 it was named *Borrelia burgdorferi* in honor of its founder. Since that time 18 etiological agents have been discovered (16), which were merged into *Borrelia burgdorferi* sensu lato complex and can be transmitted from different animal

hosts to humans via tick vector. Some of these species cause Lyme borreliosis, the most frequent tick-borne infectious disease in the northern hemisphere (17).

Along with *B. burgdorferi* s.l. complex, that includes genospecies vectored by hard ticks and may cause Lyme borreliosis, there are two more groups of *Borrelia* genospecies (18) that are referred to relapsing fever (RF). It is believed that the history of relapsing fevers goes down to ancient Greece and Egypt (19) but the relations between this disease and a tick bite had not been revealed until the beginning of the 20th century (19). Since then even more relapsing-fever agents of *Borrelia* genus have been specified (20). A group of RF *Borrelia* spirochetes, such as *B. duttonii, B. hermsii* and *B. recurrentis*, are causative agents of relapsing fever using soft-bodied ticks or lice (for *B. reccurentis*) as vectors (21). Other genospecies, *B. theileri, B. lonestari* and *B. miyamotoi*, like *B. burgdorferi* s.l., use hard-bodied ticks as vectors but are genetically closely related to RF group (22). *Borrelia miyamotoi* was first found in Japan in *I. persulcatus* ticks (23) and since then it has also been found in ticks in Europe (24, 25) and America (26-28). Recently, the human cases of a disease, accompanied with fever and headache and caused by *B. miyamotoi* were reported (29-32).

1.2. Zoonoses and vector-borne diseases

The majority of emerging and reemerging diseases and at least 60% of all human diseases are caused by viruses, bacteria, protozoa, fungi and helminths that can be transmitted from wild or domestic animals to humans (33). These diseases are called zoonoses and for the most of them humans are considered to be the "dead-end". The most known zoonotic diseases include type A influenza, rabies, anthrax, Ebola hemorrhagic fever, leptospirosis, toxoplasmosis, etc. Some of the zoonotic diseases are not transmittable to humans directly (via scratches, bites, aerosol, etc.) but need a vector, usually an arthropod (insects, spiders, ticks etc.). The vector acquires an infection while feeding on an infected reservoir animal and then transmits it to a human with next feeding. The most known example of a vector-borne zoonotic disease, which has strongly influenced the history of civilizations, is bubonic plague, that has killed over 100 million people in the medieval Europe, is caused by *Yersinia pestis* that is associated with rats and fleas (34).

The term "tick-borne pathogens" comes for the group of etiological agents, i.e. viruses, bacteria, protozoa, that use ticks as vectors. In Europe, one of the most common tick-borne pathogens is the virus that causes tick-borne encephalitis – an infectious disease that affects the central nervous system and can lead to long-term neurologic sequelae or death (35). Ticks also transmit bacteria of Anaplasmataceae family, causing human granulocytic anaplasmosis (36), and *Rickettsia* spp. bacteria, the causative agents of typhus and spotted fever in humans. However, Lyme borreliosis is the most common tick-borne

disease in the northern hemisphere with 85 000 cases registered in Europe (37) and up to 30 000 in North America annually (38).

1.3. Taxonomy, structure and morphology of *Borrelia*

1.3.1. *Borrelia* genus

Borrelia is a single cell bacterium that belongs to Spirochaetes phylum that contains a single class Spirochaetes, a single order Spirochaetales and three families – Brachyspiraceae, Leptospiraceae and Spirochaetaceae. The last one consists of genera *Borrelia* and *Treponema* representing the causative agents of Lyme borreliosis or relapsing fever, and syphilis, respectively.

Spirochetes are chemoorganoheterotrophs which means that they utilize energy from organic sources, such as carbohydrates, lipids, and proteins to fix carbon and form their organic compounds.

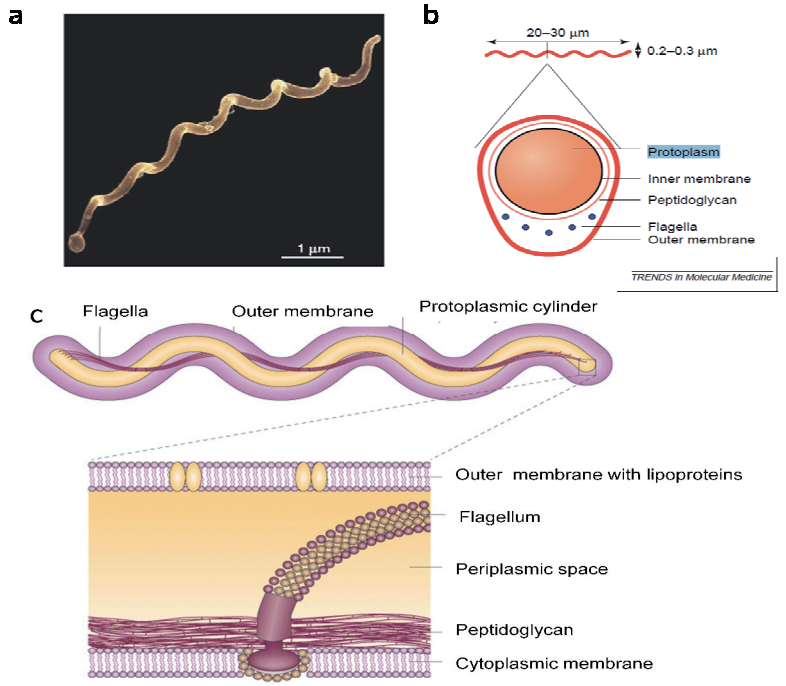

Figure 2. The structure of *Borrelia* spirochete. a – Electron micrograph of *B. burgdorferi* (39). b – *Borrelia* is a flat, coiled, 20–30 µm long and 0.2–0.3 µm wide bacteria. Its protoplasm is surrounded by the inner membrane and peptydoglycan layer (40). c – The flagella are twisted along the bacteria in the periplasmatic space between peptidoglycan and outer membrane. Flagella anchor in the inner membrane. (Image sources: panels a and c (39), panel b (40)).

Borrelia spirochetes are long, thin, helically coiled wave-like double-membrane bacteria 0.2 to 0.5 μm wide and 10 to 30 μm long (41, 42). A spirochete's cell consists of three major compounds: protoplasmic cylinder, axial filaments (flagella) and outer membrane (19) (Figure 2). It has recently been proposed that *Borrelia* are neither Gram-negative nor Gram-positive but may belong to their own, separate eubacterial phylum (43). *Borrelia* are more closely associated with Gram-negative bacteria as they have double-membrane structure. However, the cytoplasmic membrane is closely associated with peptidoglycan layer – a typical feature of Gram-positive bacteria (44). Apart from Gram-negative bacteria, the borrelial outer membrane is fluid, unstable, devoid of lipopolysaccharides but with an abundant number of lipoproteins (44-46).

The spirally coiled protoplasmic cylinder is surrounded by a complex of plasmamembrane on the outside and cytoplasm in the inner side (39). Axial filaments consist of two or more flagella protruding from the opposite poles of the cell through pores and twining around the cylinder between peptoglycan layer of the flexible cell wall and outer membrane (19, 47). Lyme *Borrelia* has up to 11 flagella in its axial filaments (15), while RF genospecies might have up to 30 (19). The rotation of axial filaments drives bacteria forward by producing screw-like motions: rotating around its axis, the cell "bores" into liquid getting translational motion (47). The protoplasmic cylinder contains a linear chromosome and multiple linear and circular plasmids (48). Under unfavorable conditions spirochetes form cysts, the locomotion processes stop and metabolism slows down (49). With conditions improving, the cyst wall breaks and all vital processes recover (50).

1.3.2. Genetic characteristics

Borrelia spirochetes are the only known members of the Spirochaetes phylum that have linear DNA molecules. The *Borrelia* genome consists mainly of a linear chromosome that is about 1×10^6 basepairs long, of which about 90% comprise coding sequences but also intergenic sequences and RNAs. The chromosome carries the majority of housekeeping genes and appears to be constant in gene content and organization (45). *Borrelia burgdorferi* has a unique structure of ribosomal RNA gene set, that consists of one copy of 16S rRNA (*rrs*) gene and two copies of each 5S and 23S rRNA *(rrl-rrf*). These genes and intergenic spacer regions between them are commonly targeted to detect *Borrelia* in a tick, mammal as well as a human specimen. The 5S-23S rRNA gene is, however, present only in the *B. burgdorferi* s.l. complex genospecies. That makes it a good target to distinguish Lyme *Borrelia* from relapsing fever group genospecies, while *glpQ* gene is present only in the genome of RF group *Borrelia*. Other chromosomal genes used for the detection and identification of *Borrelia* are, for example, *p66* (outer membrane spanning protein) and flagellin genes *flaA* and *flaB*.

Borrelia has also an extra-chromosomal genome that consists of plethora of both linear and circular plasmids in the size range from 5 to 220 kb (51). It has been shown that almost 90% of *B. burgdorferi* s.l. plasmids are not homologous to the other microorganisms' plasmids, characterized hitherto (48). Plasmids encode mostly differentially expressed surface proteins that interact with *Borrelia*'s vertebrate and arthropod hosts (51). Plasmids demonstrate greater variability of gene content and have a various representation among different strains (45). Of the plasmid-encoded genes, *ospA* and *ospC* are often targeted in the detection of *Borrelia* genospecies.

1.4. *Borrelia* genospecies

There are 36 genospecies known in the *Borrelia* genus, including those of *B. burgdorferi* s.l. group and relapsing fever *Borrelia* group. Spirochetes of *B. burgdorferi* s.l. or Lyme borreliosis complex are distributed worldwide, except Australia, and are vectored by hard-bodied (Ixodidae) ticks. Relapsing fever *Borrelia* that use soft (Argasidae) ticks or lice as vector are most common in Africa, those that are vectored by hard ticks are distributed in North America and Europe (52).

1.4.1. The Lyme *Borrelia* genospecies

The Lyme *Borrelia* spirochetes include at least 18 genospecies (16). Of them, only four are known certainly to cause a disease – *B. afzelii, B. garinii*, *B. bavariensis* and *B. burgdorferi* s.s. (16, 53). All four are distributed in Europe but *B. burgdorferi* s.s. is the only one responsible for Lyme borreliosis in North America (16, 54). There have been reports of detection of *B. bissettii, B. lusitaniae, B. valaisiana* and *B. spielmanii* in patients (16). The tick vector, reservoir host, pathogenicity and clinical implications (if known) of most familiar *Borrelia* species of both Lyme and relapsing fever groups found in Europe are presented in Table 1.

1.4.2. Relapsing fever genospecies

The relapsing fever group includes at least 12 genospecies associated with tick-borne relapsing fever in humans. Most of RF spirochetes are transmitted by soft-bodied ticks, however, up to now there are three genospecies known as vectored by *Ixodes* ticks in North America and in Europe.

Table 1. *Borrelia* genospecies of Eurasia, vectors, host range and pathogenecity

Genospecies	Vector	Reservoir host	Disease	Reference
B. burgdorferi s.s.	*Ixodes scapularis* *I. pacificus* *I. ricinus*	Rodents, birds, lizards, big mammals	EM, arthritis, cardiologic disturbances, NB	(55, 56)
B. afzelii	*I. ricinus* *I. persulcatus*	Rodents	EM, arthritis, cardiologic disturbances NB	(57)
B. garinii	*I. ricinus* *I. persulcatus* *I. uriae* *I. hexagonus* *I. nipponensis* *I. pavlovskyi* *I. trianguliceps*	Birds, seabirds, rodents	EM, arthritis, cardiologic disturbances, NB	(55, 58-62)
*B. bavariensis***	*I. ricinus*	Rodents	*	(63)
B. bissettii	*I. ricinus* *I. scapularis* *I. pacificus* *I. minor*	Rodents, birds	*	(64)
B. spielmanii	*I. ricinus*	Rodents	EM	(65, 66)
B. lusitaniae	*I. ricinus* *I. persulcatus*	Lizards, rodents	*	(67)
B. valaisiana	*I. ricinus* *I. persulcatus* *I. granulatus*	Birds, rodents, lizards	*	(68, 69) (70)
B. kurtenbachii	*I. scapularis*	Rodents	Potentially pathogenic	(71)
Relapsing fever group genospecies				
B. miyamotoi	*I. ricinus* *I. persulcatus* *I. dentatus*		LB-like symptoms with relapsing fever	(72) (23, 73)

* Isolations from patients.

** Formerly *B. garinii* OspA serotype 4, *B. garinii* ribotype IV, rodent-associated *B. garinii*. Recently it has been found that *B. garinii* NT29 is genetically more closely related to *B. bavariensis* (74). In the current thesis and in the articles *B. bavariensis* is referred to as identical to *B. garinii* PBi, while *B. garinii* NT29 is assigned to *B. garinii* subgroup NT29 or Asian type.

1.5. The infection cycle

Ticks and vertebrates are essential for maintaining *Borrelia* in the natural foci. The infection cycle of *B. burgdorferi* s.l. as well as RF group spirochete *B. miyamotoi* involves hard-bodied ticks of the *Ixodes* species as well as various vertebrate hosts. Mammals, primarily small rodents and birds serve as long-lasting natural reservoirs for the spirochetes while ticks play role both as reservoirs and as vectors.

1.5.1. Transmission of *Borrelia*

Ticks feed once at each of the three development stages – larvae, nymph and adult – and can acquire *Borrelia* infection from infected rodent at each blood meal. In the case of *B. burgdorferi* s.l. transovarial transmission (from adult female to her offspring) is a rare event (75). Also male ticks do not usually need blood meal (76, 77) and females prefer to feed on large-sized mammals that are not usually competent hosts (78). Thus adult ticks play insignificant role in the maintenance of *B. burgdorferi* s.l., whereas larvae and nymphs are most important in the circulation of *Borrelia* in the natural foci. If a larva becomes infected during its first blood meal, the infection persists in the tick during molting into next development stages – to nymph and then to adult. Infected nymph therefore transmits the infection to the mammal host while feeding. Moreover, if the mammalian host is infected, the nymph can acquire a new infection and become infected with multiple *Borrelia* genospecies or other tick-borne pathogens. After feeding, the nymph molts into an adult with infection(s) maintaining from the previous blood meals.

It has been shown that infection of ticks with *Borrelia* during co-feeding is also effective (79, 80). In this case an infected nymph should feed with uninfected larvae or another nymph on the same animal in the closest proximity and in the same time. No systemic infection develops in the mammalian host. Spirochetes are transmitted into the blood of mammal from salivary glands of infected nymph and are digested with the mammal´s blood by larvae and/or nymphs attached in the same site.

Transovarial transmission (from infected female to the offspring) of *Borrelia* spirochetes has been demonstrated for RF group genospecies, *B. miyamotoi* (27). In case of *B. burgdorferi* s.l. transovarial transmission is very rare, if not absent (81).

It has also been shown by Alekseev, that *Borrelia* spirochetes, particularly *B. garinii*, *B afzelii* and *B. burgdorferi* s.s. are transferred from an infected male to a female during copulation, however no infection in the opposite direction, i.e. from the infected female to a male, has been shown (82).

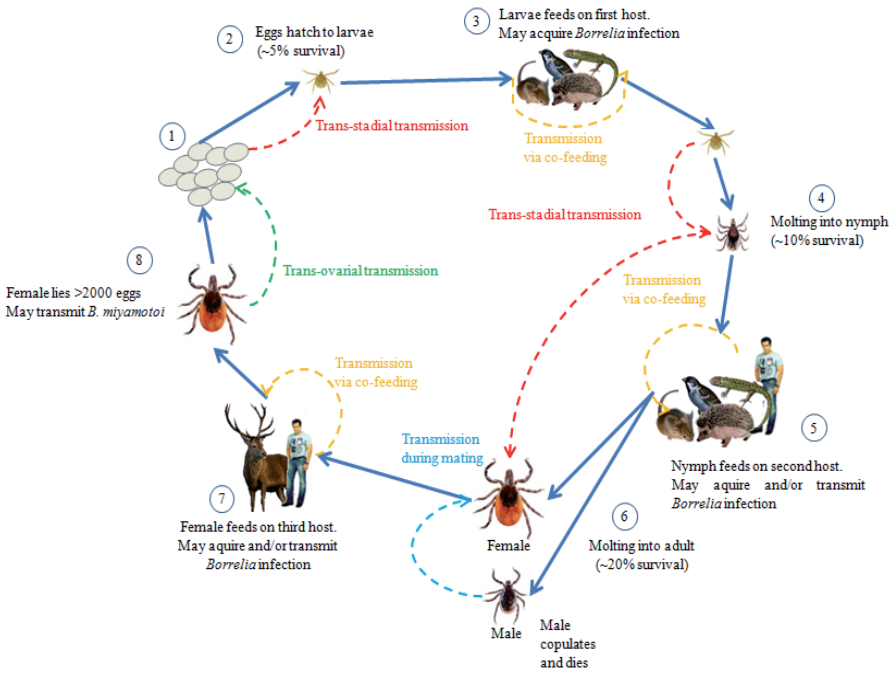

Figure 3. Maintaining and transmission of *Borrelia* infection within the life cycle of *Ixodes* sp. tick.

1.6. Tick vectors

1.6.1. Tick vector species

Ticks of the *Ixodes* species are the main vectors of *Borrelia* spirochetes as well as other tick-borne pathogens. In North America *Borrelia* spirochetes are transmitted by *I. scapularis* and *I. pacificus*, while in Eurasia *I. ricinus* and *I. persulcatus* are associated with the transmission of these pathogens (76). *Ixodes ricinus* is the most common vector for tick-borne pathogens in Europe, while *I. persulcatus* is the main vector in Asia. Estonia is situated in a unique region in the Eastern Europe, where ranges of both tick species overlap.

In the temperate climate zone ticks' activity period lasts from April until the end of October, when the average temperature is above 5 degrees centigrade.

It has been shown that under conditions of sympatry both *I. ricinus* and *I. persulcatus* are vectors of *B. burgdorferi* s.l. as well as *B. miyamotoi* (83). However, several studies have indicated that on the whole, *I. persulcatus* is a more effective vector of *Borrelia* (83) as well as of tick-borne encephalitis virus (1, 84) than *I. ricinus*.

1.6.1.1. Ixodes ricinus

The area of *I. ricinus* ticks distribution lies from Portugal to Middle Asia and from Middle East and North coast of Africa to the central regions of Sweden and Finland (Figure 4).

This tick species prefer humid, lowland moraine landscapes with broadleaved forests rich in vegetation (83). The seasonal abundance of *I. ricinus* ticks has two peaks: in early summer and in early autumn.

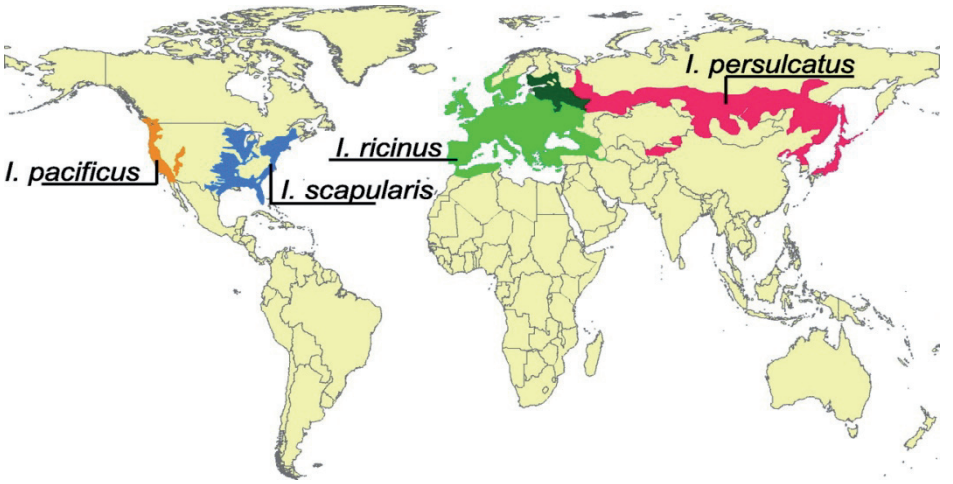

Figure 4. Approximate geographic distribution of main medically important ticks of the *Ixodes* sp. (85).

1.6.1.2. Ixodes persulcatus

Ixodes persulcatus, or the taiga tick, is common in the Asian countries, Central and Southern regions of Russia and in the Baltic region (Figure 4).

The species occurs in upland landscapes with taiga-type dark coniferous forests or secondary forests, replacing them (83). *Ixodes persulcatus* is more cold resistant and becomes active earlier than *I. ricinus*. Host-seeking adults are more aggressive, compared to *I. ricinus* (86). The activity peak for *I. persulcatus* is in mid-spring.

1.6.1. Tick's development cycle

Ixodes ticks have four development stages: egg, larva, nymph and adult. This is a three-host tick species, as larvae, nymphs and adults each have a single blood-meal on different animal host. After feeding, they detach from their host. Subadult ticks then undergo molting into the next development stage, and adult

females lay eggs. Generally, *I. ricinus* and *I. persulcatus* complete their development cycle in 3 years. However, in more favorable conditions it may take less time and otherwise, in unfavorable conditions development cycle may last up to 6 years (87).

The newly-hatched larvae seek for a host in the lower vegetation layer, preferring insectivores (hedgehogs, shrews), rodents, reptiles and birds. They feed during 2–5 days and when engorged (the weight of larvae can increase to 10–20-fold), fall into the vegetation and molt into the next development stage. Nymphs seek for a host in the upper vegetation layer and prefer to feed on small animals, but also on medium-sized mammals, such as hares and even roe deer. When questing for a host, ticks usually use the so-called "ambush" strategy: they crawl up on the grass stems or climb on the edges of leaves near the ground, extend their first pair of legs and wait for a host to pass. *Ixodes ricinus* and *I. persulcatus* have no eyes. For host detection they use Haller's organ, which is located on the terminal segment on the each of two first legs. This organ is responsible for sensing the smell (olfaction), vibrations, temperature, humidity and carbon dioxide. Nymphs stay attached to the animal for 4–7 days until engorged, and then molt on the vegetation into adult male or female. Adult ticks climb higher on the vegetation, up to about 1 m (88) and thus restrict themselves to feeding on medium- and large-sized animals (89). During the blood-meal, lasting for 6–13 days, an adult female can increase its weight up to 100-fold (77). Males are considered as facultative feeders as they do not need a blood-meal to fertilize female (77). However, they may take small blood-meals while remaining on a host in search of females (76). Copulation may take place on vegetation as well as on a host, while the female is feeding. A male can fertilize numbers of females, if they are close in space. After copulation males die. When mated, a female lays in vegetation a single batch that consists of over 1000 eggs, and then dies (76).

Ixodes ticks are sensitive to temperature and humidity fluctuations. Questing for a suitable host may take up to several weeks during which ticks are highly exposed to environmental changes. To avoid desiccation, ticks need to interrupt active questing and descend into lower vegetation layer near the soil, where relative humidity is over 80%. That, however, decreases the chances for host finding.

1.6.2. Tick on a host

Having found a suitable place for suction, ticks cut animal's skin with mouthparts called chelicerae and insert the hypostome, a chitin tube-like organ with recurved teeth. In order to be "fixed", the salivary glands produce a glue-like secretion that sticks the hypostome into the skin.

When an uninfected tick is feeding on a host with *Borrelia* infection, spirochetes migrate into the tick midgut with a bloodmeal, where they remain localized during the tick's lifespan. However, it has been shown that concentration of spirochetes decreases 10-fold during each molting into next stage (90) and with time after detachment. Thus, at the time of next questing the concentration of *Borrelia* spirochetes in tick is least.

Borrelia spirochetes must have a high adaptive capacity to survive in the different environment conditions: in arthropod system with no immune response as well as under the pressure of the animal host immune system. When *Borrelia*-infected tick starts feeding on a host, spirochetes began to actively multiply in the midgut (90). It has been shown that spirochetes transmission efficiency is dependent on time: the risk of infection increases when tick is feeding over 24 hours and becomes high after 36 hours of feeding (91). About 48 hours after tick's attaching, spirochetes migrate into the host's blood (92). To be efficiently transmitted, spirochetes must pass the midgut epithelium through hemolymph to the salivary glands. *Borrelia* triggers the expression of outer membrane lipoprotein A (OspA) (93) that binds to the respective tick receptor –TROSPA, enabling adherence to the tick midgut epithelia (94). When the incoming blood changes the environment in the midgut, *Borrelia* downregulates the expression of OspA and increases the expression of OspC, a lipoprotein essential for the initial infection. OspC binds to a tick salivary protein Salp15, which has immunosuppressive properties and inhibits antibody-mediated killing of *B. burgdorferi* spirochetes by the host immune system (95). The regulation of expression of OspA and OspC proteins is dependent on environment pH and temperature, and correlates with the migration of spirochetes from tick to host. Also, the plasminogen of the host blood is able to bind with *Borrelia* plasminogen-binding proteins and mediate the extracellular proteolytic activity, enabling the dissemination of spirochetes in the host tissues (96-98).

1.7. Reservoir hosts

Animals have their role in maintaining the tick population by providing a blood meal but may also be a source of infection. They are responsible for the spreading of ticks as they transfer ticks attached to their bodies over distances. Migratory birds may also play a role in long-distance dispersal of ticks and therefore may introduce ticks as well as tick-borne pathogens into new geographical foci (99). Animals harboring the infection are competent reservoir hosts and represent a long-term infection source for ticks, feeding on them (100). In the context of *Borrelia*, reservoir animal hosts mostly do not develop symptomatic infection (101).

The main reservoir hosts in Europe for *B. burgdorferi* s.l. and *B. miyamotoi* are rodents: mice, voles, rats and squirrels (66, 78, 102-108). The host competence has also been demonstrated by insectivore species, such as shrews and

hedgehogs (109-111). Also, passerine birds, especially blackbirds and thrushes (112, 113) and pheasants (114) have also been shown to be reservoirs for LB genospecies, *B. garinii* and *B. valaisiana*. Small-sized animals represent the major blood meal source for larvae and nymphs and thus are most important for circulation of *Borrelia* in the natural foci. Large-sized mammals, such as deer, moose, cattle and sheep, are considered non-significant reservoirs (89, 115) because adult ticks mostly feed on them. Incompetent hosts may participate in circulation of *Borrelia* by supporting the transmission via co-feeding (116). Information on the most prevalent *Borrelia* genospecies in Europe and their hosts is presented in Table 2.

Table 2. Reservoir hosts of the most common *Borrelia* genospecies in Europe

	Rodents		
Wood mouse	*Apodemus (Sylvaemus) sylvaticus*	*B. afzelii*	(117, 118)
Black-striped mouse	*Apodemus agrarius*	*B. afzelii*	(102)
Yellow-necked mouse	*Apodemus flavicollis*	*B. afzelii, B. garinii**	(103)
Bank vole	*Myodes glareolus*	*B. afzelii, B. garinii**	(103)
Meadow vole	*Microtus agrestis*	*B. afzelii*	(78)
Edible dormice	*Glis glis*	*B. spielmanii*	(104, 105)
Hazel dormice	*Muscardinus avellanarius*	*B. spielmanii*	(106)
Garden dormice	*Eliomys quercinus*	*B. spielmanii*	(66)
Red squirrel	*Sciurus vulgaris*	*B. afzelii, B. burgdorferi s.s*	(108)
	Insectivores		
European hedgehog	*Erinaceus europaeus*	*B. afzelii*	(119)
	Lagomorphs		
Brown hare	*Lepus europaeus*	*B. burgdorferi s.l.*	(110)
Mountain hare	*Lepus timidus*	*B. burgdorferi s.s.*	(120)
	Birds		
Common blackbird	*Turdus merula*	*B. garinii, B. valaisiana*	(121)
European thrush	*Turdus philomelos*	*B. garinii, B. valaisiana*	(121)
Pheasant	*Phasianus colchicus*	*B. garinii, B. valaisiana, B. burgdorferi s.s*	(114)
Razorbill	*Alca torda*	*B. garinii*	(122, 123)
Puffins	*Fratercula arctica*	*B. garinii*	(124)
	Lizards		
Common wall lizard	*Podarcis muralis*	*B. lusitaniae*	(125, 126)
Sand lizard	*Lacerta agilis*	*B. lusitaniae*	(126)

* May be referred to as *B. bavariensis*, that was previously named *B. garinii* (ospA serotype 4, NT29 biotype, NT29-like).

Figure 5. Competent reservoir hosts of *B. burgdorferi* s.l. All images are courtesy of Josef Lubomir Hlasek (Source: www.hlasek.com, retrieved 03.08.2013).

1.8. Epidemiology

Lyme borreliosis is the most abundant vector-borne disease in North America and in Europe, with more than 30 000 and 60 000 reported cases each year, respectively (45). The Baltic States and Southern Sweden represent the highest incidence numbers in the Northern Europe (127). In Estonia the annual incidence rate of LB increased dramatically over the last decade, from 23.5 per 100 000 population in 2002 to 171.8 cases per 100 000 in 2011 (128) (Figure 6). In 2012 LB incidence rate in Estonia established 115,4 cases per 100 000 population. According to the data retrieved by the Health Board, the most endemic regions in Estonia are Saaremaa and Hiiumaa islands, and Läänemaa county in the mainland (Figure 7). However, it should be noted that on the whole the LB morbidity on the islands is at least twice as high as in the mainland regions. Although cases are diagnosed all year round, most patients are reported from mid-summer until mid-autumn.

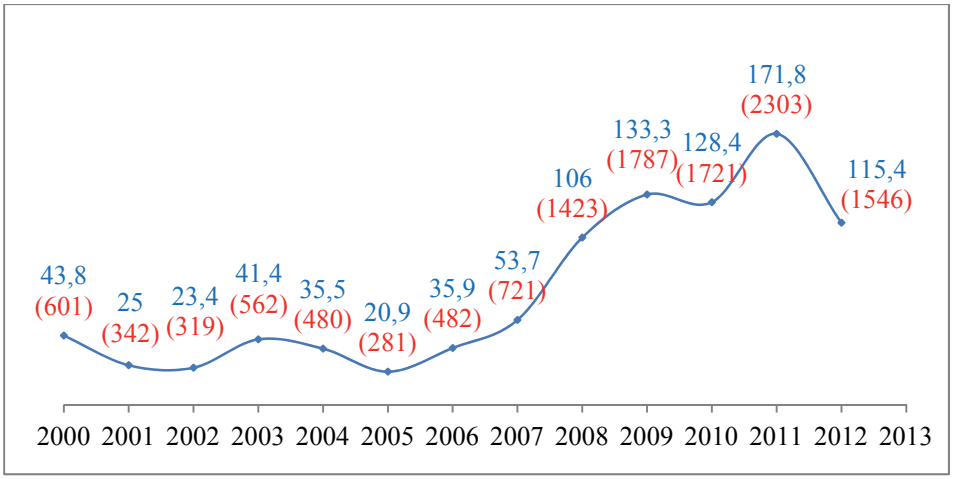

Figure 6. Lyme borreliosis in Estonia in 2000–2012. The graph demonstrates the incidence rates of Lyme borreliosis per 100 000 population and the number of registered cases (in parenthesis in red).

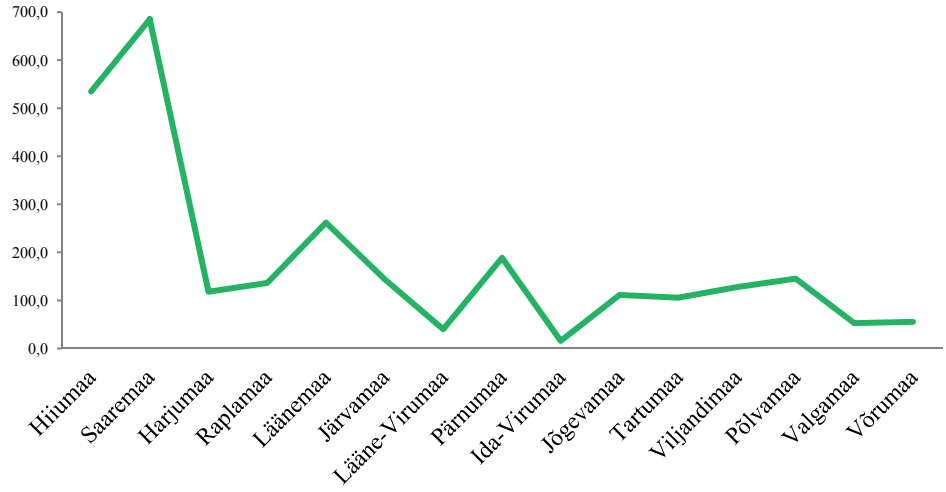

Figure 7. The most endemic regions in Estonia are Hiiumaa, Saaremaa, Läänemaa and Pärnumaa. The graph demonstrates the incidence rate per 100 000 population in the counties of Estonia in 2012.

There have been few reports regarding the prevalence rates of *B. burgdorferi* s.l. in ticks. The study of Mäkinen et al. reports a 15% infection rate of *B. burgdorferi* s.l. in *I. ricinus* ticks fromVormsi island (129). The presence of two *Borrelia* genospecies, *B. garinii* and *B. afzelii*, in Estonian ticks have been reported by Mäkinen and Postic (129, 130). According to Rauter and Hartung Estonia is situated in the region of low infection rates (nymphs ≤ 11%, adult ticks ≤ 20%) (131).

1.9. Clinical manifestations

1.9.1. Lyme borreliosis

Once inoculated into the skin at the site of a tick bite, *Borrelia* spirochetes may disseminate into various organs and tissues (132). Lyme borreliosis is a multisystemic, multistage inflammatory disease. However, the clinical manifestations seem to be different in Europe comparing to North America that might be due to the different genospecies of *Borrelia*, circulating in these geographical areas. In North America, where *B. burgdorferi* s.s. is the only genospecies causing LB, arthritic disorders are more frequent as *B. burgdorferi* s.s. is particularly arthritogenic and may cause chronic, antibiotic-refractory arthritis (133). In contrast, in Europe, where *B. afzelii* and *B. garinii* serve as the main cause of the disease, late skin manifestations and neurological disorders have been reported frequently (134). Here and later on in the text, only the European cases of LB will be regarded.

Traditionally, LB course is divided into different stages (135). However, the division is rather conditional because most patients do not develop all the stages and manifestations, and often undergo early stages asymptomatically (136).

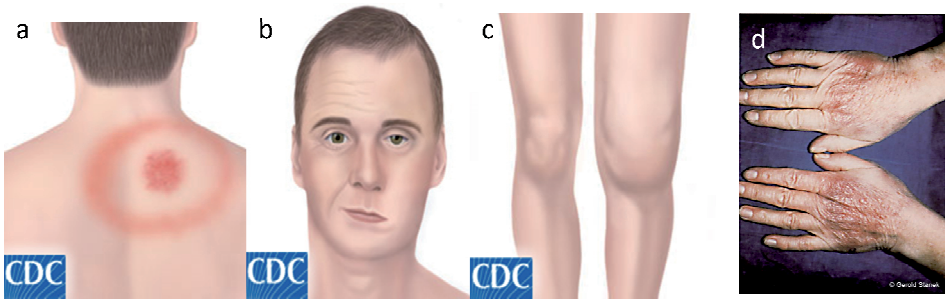

Figure 8. Clinical signs of Lyme borreliosis: a – erythema migrans, b – facial palsy at neuroborreliosis, c – Lyme arthritis, d – acrodermatitis chronic atrophicans. (Image sources: panels a – c http://www.cdc.gov/Lyme/signs_symptoms/ , panel d – http://meduni09.edis.at/eucalb/cms_15/index.php?option=com_content&task=view&id=99&Itemid=110 ; retrieved 03.08.2013).

The first and the most frequent LB sign is a development of a characteristic skin lesion, called erythema migrans (EM), that appears during 2–30 days after tick bite at the site of a bite (127). Traditionally, this lesion is annular, red colored with a clearance in a centre, but can also be homogenous (137). Local itching or burning as well as fatigue and headache may be accompanied during the enlargement of skin lesion (138). EM is the basis of primary LB diagnosis, which can be provided by physician observation, as serological diagnostics are not essential at this stage (138). Several EM indicate on a systemic dissemination (139).

Another dermatologic manifestation of LB is borrelial lymphocytoma (BL) that may appear weeks to months after bite in the proximity to the bite site (139). This bluish-red swelling is located often on the nipple or ear lobe (140).

Late skin manifestation of LB, which develops months to years, is acrodermatitis chronica atrophicans (ACA) – a bluish-red discoloration with edema on the elbow, knee, hand or feet extensor sites (139). This symptom is caused primarily by *B. afzelii* (141). ACA does not resolve unless properly treated with antibiotics (142). The conditions that may also be associated with ACA include arthralgia, polyneuropathy and fatigue (142).

The infection of the nervous system with *Borrelia* is usually acute and rarely chronic. Neurological symptoms may arise 1–12 weeks after tick bite (143) and are associated mostly with *B. garinii* (144). Neuroborreliosis (NB) usually includes radiculoneuritis, which can be accompanied with mild meningeal symptoms, and cranial neuropathy (145). The most common meningeal symptoms involve headache and neck inflexibility. These are rare and usually mild, however, some patients may experience more intense pain. Other involvements of nervous system in NB include cranial nerve palsies, encephalitic and encephalomyelitic symptoms (133).

Joint damages, or arthritis, due to LB in Europe are less frequent, compared to that in North America (133, 146). About 0.5% of European LB patients experience cardiological abnormalities that can appear within two months after tick bite and come along with NB or EM (147). Another rare manifestation of LB is eye tissue and/or nerve involvements, which can include conjunctivitis, optic neuritis, keratitis as well as extraocular involvements due to cranial nerve paresis (148, 149).

1.9.2. Tick-borne relapsing fever

As the name of the disease indicates, relapsing fever is characterized by episodes of fever which may recur or relapse for several times. Although tick-borne relapsing fever (TBRF) cases in humans due to such *Borrelia* genospecies as *B. hermsii*, *B. parkeri* and *B. hispanica* are well known and described, the disease caused by *B. miyamotoi* was reported and described for the first time in 2011 (29).

After an incubation period that lasts about two weeks after tick bite patients experience viral-like symptoms which include high fever, chills, headache, vomiting, fatigue and myalgia (29, 30, 150). According to Platonov et al. (29) *B. miyamotoi* infection reveals more systemic manifestations than *B. garinii*, as well as higher body temperatures. Fever lasts for 2–5 days and commonly relapses 1–2 times within 2 days to 2 weeks. *B. miyamotoi* infection has also shown functional impairments of the liver, kidney, heart and other organs (30).

As *B. miyamotoi* is a RF group *Borrelia*, it might also lead to neurologic, ocular, respiratory, and pregnancy implications that are associated with relapsing fever (29).

1.10. Laboratory diagnostics

The diagnosis of a LB onset traditionally goes with observation of patient's clinical symptoms, as well as history of probable tick exposure. All the stages and clinical manifestations of LB except EM must be confirmed serologically. However, due to a large variety of genospecies causing LB in Europe, as well as clinical manifestations diversity, it is difficult to standardize the diagnostics.

The most common method for routine laboratory diagnostics is the enzyme-linked immunosorbent assay (ELISA) which is presented by plethora of commercial kits. However, the absence of common standards causes difficulties in diagnostic criteria comparing. First generation ELISAs used whole-cell sonicates as antigens that could cross-react with a number of antigens in other bacteria, as these were not unique to *B. burgdorferi* s.l. (151). The second generation ELISA test systems have improved specificity by using purified native antigens, such as flagellin. Third generation ELISAs use synthetic proteins or recombinant antigens in single antigen tests as well as in combination (152). The use of new target proteins and antigens, such as VlsE or DbpA, may improve the sensitivity and specificity of diagnostic methods and thus reduce false-positive and false-negative tests (153).

Immunofluorescent antibody assay (IFA) was an early method for testing *Borrelia* antigens in the patients' serum specimen. It used cultured spirochetes fixed onto the glass and incubated with serum, with subsequent addition of FITC-labelled anti-human IgG or IgM (151). However, this method appeared comprehensive for routine use and was commonly replaced by ELISA.

In order to improve the diagnosis accuracy it is recommended to use two-step diagnostic systems, which include ELISA or IFA followed by western blot (WB). Samples tested negative for ELISA or IFA are reported as negative, whereas positive or equivocal results should be confirmed and/or excluded as false-positive with WB, a procedure in which specific proteins, preferably recombinant antigens, are used (154).

Other possible methods such as microscopic detection, culture method, polymerase chain reaction (PCR) are not commonly used for routine diagnostics. Microscopy of spirochetes in the patients' specimen is ineffective due to difficulties in finding of appropriate tissue or body fluid with acceptable spirochete number (154). Culture is time-consuming, low-sensitive and cumbersome method because *B. burgdorferi* s.l. is hardly cultivable and fastidious about growth medium and conditions, although it can be achieved

from skin biopsies (EM, LB, ACA), synovial tissues, blood samples and cerebrospinal fluid (151). Although overall PCR is quite a sensitive method, it has but limited use in laboratory diagnostics of LB due to low spirochetemia in body fluids, such as blood or CSF, used for the initial DNA isolation that may result in low yield of trustable PCR results (151).

1.11. Treatment

As a disease of bacterial nature, LB is successfully treated with antibiotics. The early antimicrobial therapy reduces the risk of complications' development. However, the preventive antibiotic treatment prior to tick bite is not recommended because only few ticks are infected with *Borrelia* and moreover, the timely tick removal in 24h after bite reduces the possibility of *Borrelia* spirochetes dissemination in skin tissues. The main antibiotics used are amoxicillin and doxycycline as well as third-generation cephalosporines. The treatment duration is from 10 to 30 days (155).

The recently reported human cases of TBRF due to *B. miyamotoi* infection have also been shown to be successfully treated with antibiotics, such as doxycycline, ceftriaxone, amoxicillin (29). The early antimicrobial drug therapy reduces fever relapses as well as other clinical implications. A single 14 – 20 day course of antibiotic treatment appears to clear *B. miyamotoi* infection (29, 32).

2. AIMS OF THE STUDY

The main objective of the study was, as the first attempt in Estonia, to reveal, estimate and characterize *Borrelia* in tick population in different regions in Estonia. This was achieved by the following steps:

- Determination of tick-borne pathogens removed from migratory passerines during southward migration
- Investigation of the presence, prevalence, distribution and genetic characterization of *B. miyamotoi* genospecies in questing ticks in Estonia
- Investigation of the presence, prevalence, distribution and genetic characterization of *B. burgdorferi* s.l. genospecies in questing ticks in Estonia

It was also hypothesized that there might be some differences in distribution of *Borrelia* among two tick species as well as between allopatric and sympatric areas.

3. MATERIALS AND METHODS

3.1. Tick sampling

3.1.1. Ticks from the vegetation

In all, ticks were collected from 64 sites: 49 sites in 8 counties of mainland Estonia and 15 sites on the islands Muhu and Saaremaa (Figure 9). The investigation of the presence of *B. miyamotoi* was carried out in ticks collected from all 64 sites. The presence of *Borrelia burgdorferi* s.l. was investigated in ticks collected in 43 sites of 7 mainland counties and 10 sites on the islands Muhu and Saaremaa.

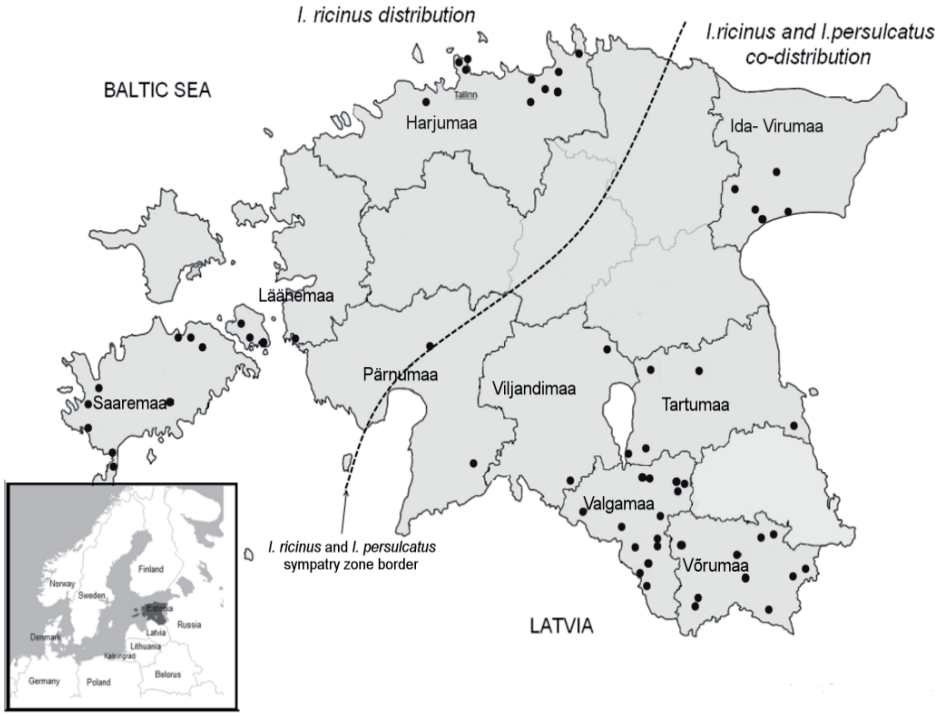

Figure 9. Tick collection places in Estonia. Ticks were collected in 64 places, represented by dots.

During 2006-2010, adult and nymphal ticks were collected from the vegetation monthly from April until November using standard flagging method. At each site a 100 m long transect line was dragged by 1 m^2 flannel cloth, with the cloth examined every 5 m. Ticks were removed from the cloth with forceps, placed in glass tubes according to sex and stage, and kept alive until further examination.

3.1.2. Ticks removed from birds

The southward migrating birds were captured by ornithologists at Kabli and Pulgoja bird stations during fall migration in August – October from 2006 to 2009. Birds trapping, identification and tick removal was performed by ornithologists. Ticks were collected from birds' heads with tweezers and placed in 70% ethanol. Within one month after tick collection, the specimens were processed individually according to the bird species they were removed from.

3.2. Morphological identification of tick species

Tick species were independently determined by morphological criteria by two entomologists using a stereo microscope. Collected ticks were then washed in 70% ethanol, rinsed twice with sterile phosphate-buffered saline (PBS) and stored individually at –70ºC. For further nucleic acid extraction, they were homogenized with TissueLyzer (Retsch, Haan, Germany) in 300 µL of sterile PBS.

3.3. Nucleic acid extraction

The nucleic acid extraction was performed in 200 µL of tick suspension with guanidinium thiocyanate-phenol-chlorophorm method (TriPure isolation system, Roche Diagnostics, Lewes, UK) following manufacturer's recommendations. Every preparation set consisted of 30 samples, including sterile water as a negative control. The extracted DNA and RNA were stored at –20ºC and –70ºC, respectively, until further use.

3.4. PCR amplification

3.4.1. Detection of *Borrelia* genospecies

All PCR amplifications used for the detection of *B. burgdorferi* s.l and *B. miyamotoi* were carried out in a reaction mix volume of 20 µL that contained 10×PCR buffer (Fermentas), 2 mM $MgCl_2$, 800 µM dNTPs, 0.5 µM of each of primers of a primer pair and 1.5 U DNA polymerase. To the PCR reaction mix, 5 µL of template DNA or the PCR product in case of nested PCR was added. The deioinized water was included in every amplification set as a negative control. To minimize contamination all the steps, including reaction mix preparation, template addition, amplification and gel electrophoresis were performed in separate rooms following sterile techniques. The PCR products were visualized in 1% agarose gel, stained with ethidium bromide (1 mg/ml). The primers' sequences used in this work are presented in Table 3.

The amplification of 1256 bp product of 16S rRNA partial rRNA gene was used for the detection of *Borrelia* presence in ticks as described previously by Fukunaga et al. (23). The first step of PCR amplification was performed with 16S-Bor-S1F and 16S-Bor-S2R primers under the following conditions: 35 cycles, 94ºC for 10 sec, 60ºC for 1 min and 72ºC for 90 sec. The nested PCR was performed as described by Fomenko (24) with inner primer pair 16S-Bor-S4F and 16S-Bor-3R. The amplification conditions included 35 cycles of initial denaturation at 94ºC, followed by an annealing step at 65ºC for 1 min and elongation at 72ºC for 90 sec.

The *B. burgdorferi* s.l. specific 5S-23S intergenic spacer region was targeted to distinguish Lyme *Borrelia* genospecies. The amplification has previously been described by Postic and Rar (156, 157). The external primer pair of NC1 and NC2 was used for the first round and internal primers NC3 and NC4 were used for the nested PCR. The cycling conditions of the first round amplification were modified as follows: 35 cycles, initiation at 94ºC for 1 min, annealing step at 58ºC for 1 min and elongation step at 72ºC for 2 min. The second round amplification was performed in 30 cycles and with annealing temperature decreased to 52ºC. The final product was 245 to 256 bp long, depending on *B. burgdorferi* s.l. genospecies.

Ticks' DNA samples positive for 16S rRNA were also amplified for *B. miyamotoi* partial *p66* (532 bp) and *glpQ* (379 bp) genes. The amplification of partial *p66* gene was performed using nested PCR with external primers M1F and M2R and inner primers M3F and M4F as was previously described (24) under the following conditions: 35 cycles, 94ºC for 5 sec, 50ºC for 10 sec and 72ºC for 30 sec. In the nested PCR the annealing and elongation times were increased to 15 and 45 sec, respectively. The amplification of *B. miyamotoi glpQ* partial gene was performed for all samples, positive for *p66*, as was described by Fomenko (24). Primers Q1F and Q2R were used for the initial amplification, that was performed in 35 cycles of 94ºC for 10 sec, 50ºC for 15 sec and 72ºC for 35 sec. The final generation of 379 bp product was achieved using primer pair Q3F/Q4R. Nested PCR annealing temperature was lowered to 52ºC and the amplification steps were reduced by 5 sec time each.

3.4.2. Detection of other tick-borne pathogens

Ticks' RNA and/or DNA samples were also used to detect the possible co-infections with other tick-borne pathogens. The detection of tick-borne encephalitis virus by real-time PCR and partial E gene amplification are described in Papers I and II. Briefly, real time reverse transcriptase PCR modified after Schwaiger and Cassinotti (158) was performed for the initial screening of TBEV with primers F-TBE1 and R-TBE1 as well as probe TBE-probe-WT. The amplification was performed in a 25 µl reaction mixture, containing 2× Reaction mix, 0.5 µl of SuperScript III Platinum One-Step Taq

Mix (Invitrogen, USA), 0.3 µM of forward primer F-TBE1, 0.9 µM of reverse primer R-TBE1, 0.25 µM of TBE-probe-WT and 5 µl of each sample RNA. The reverse transcription was performed in 30 cycles at 42ºC and final denaturation for 10 min at 94ºC, followed by 45 cycles for 15 sec at 95ºC and 1 min at 60ºC. The 7500 Fast Real Time PCR system (Applied Biosystems) was used for PCR reactions and fluorescent detections. The positive samples were then amplified for the partial E gene as described by Skarpaas (159) with cDNA synthesis with primers 283F1 and 827R1, and final 465 bp product was amplified with primers 349F2 and 814R2. RT-PCR reaction mix consisted of 2× Reaction mix (Invitrogen, Carlsbad, USA), 0.2 µM of forward and reverse primers, 1 µl of SuperScript® III RT/ Platinum® Taq Mix (Invitrogen, Carlsbad, USA), and 5 µl of template RNA. The reaction was carried out in a 25 µL total volume. The cDNA synthesis was performed under the following conditions: 30 min at 50ºC, denaturation for 2 min at 94ºC. The touch-down program was as follows: 40 cycles, 20 sec at 94ºC, 1 min at 60ºC, and 1 min at 68ºC, followed by a final extension at 68°C for 5 min. The nested PCR amplifications were performed in a total volume of 50 µl consisting of: GeneAmp 10xPCR buffer II, 1.5 mM $MgCl_a$, 0.15 mM of each dNTP, 0.5 mM of each 349F2 and 814R2 primers, AmpliTaq DNA polymerase (2.5U) (Applied Biosystems, Roche, Branchburg, New Jersey, USA), and 5µl of target cDNA from the first PCR reaction. The cycling conditions included initial denaturation for 2 min at 94ºC, followed by 30 cycles for 1 min at 94ºC, 1 min at 65ºC, and 1 min at 72ºC, and a final extension at 72°C for 10 min.

The detection of *Anaplasma phagocytophilum* in ticks removed from birds is introduced in Paper I. The screening of *A. phagocytophilum* was performed by real-time PCR according to (160). Briefly, 5 µL of template DNA was added to the 20 µL of amplification mix, containing TaqMan Universal Master mix (Applied Biosystems, Branchburg, New Jersey, USA), 0.9 µM of each primer ApMSP2f and ApMSP2r, and 0.2 µM of ApMSP2-FAM probe. The AmpErase enzyme activation was achieved by preincubation for 2 min at 50ºC. The following touch-down program comprised 45 cycles at 95ºC for 15 sec (denaturation) and at 60ºC for 1 min (annealing-extension).

3.4.3. Ticks' species confirmation

In order to confirm the morphological tick species definition, tick DNA samples were analyzed for mitochondrial partial 16S rRNA gene PCR (161) using primers 16Sa and16Sb. The reaction was performed in a total volume of 50 µL. The Reaction mix included 10xPCR buffer, per 1.5 mM of $MgCl_2$ and dNTPs, 0.5 µM of each primer and 5U TaqPol. Cycling conditions were as follows: 95ºC for 1 min, 49ºC for 1 min, 72ºC for 2 min, 95ºC for 1 min, 47ºC for 1 min, 72ºC for 2 min, 40 cycles (95ºC for 30 sec, 45ºC for 1 min and 72ºC for 2 min), 72ºC for 10 min.

Table 3. PCR primers' sequences used for the detection and identification of *B. burgdorferi* s.l., *B. miyamotoi*, TBEV, *A. phagocytophilum* as well as for the confirmation of *Ixodes* spp

Target organism, gene/region	Primer name	5'-3' sequence	Reference
Borrelia spp., 16S rRNA	16S-Bor-S1F	GCTGGCAGTGCGTCTTAAGCATGC	(23)
	16S-Bor-S2R	CGGGTTAGAATAATAGCTTCGGG	
	16S-Bor-S3R	GTGACGGGCGGTGTGTACAAGGCCC	(24)
	16S-Bor-S4F	GAACGGGTGAGTAACGCGTGGATGATC	
B. burgdorferi s.l., 5S-23S rRNA intergenic spacer region	NC1	CCTGTTATCATTCCGAACACAG	(157)
	NC2	TACTCCATTCGGTAATCTTGGG	
	NC3	CTGCGAGTTCGCGGGAGA	(156)
	NC4	TCCTAGGCATTCACCATA	
B. miyamotoi, p66	M1F	TTCTATATTTGGACACATGTC	(24)
	M2R	CAGATTGTTTAGTTCTAATCCG	
	M3F	CTAAATTATTAAATCCAAAATCG	
	M4R	GGAAATGAGTACCTACATATG	
B. miyamotoi, glpQ	Q1F	CACCATTGATCATAGCTCACAG	
	Q2R	CTGTTGGTGCTTCATTCCAGTC	
	Q3F	GCTAGTGGGTATCTTCCAGAAC	
	Q4R	CTTGTTGTTTATGCCAGAAGGGT	
TBEV, 3' non-coding region,	F-TBE1	GGGCGGTTCTTGTTCTCC	(158)
	R-TBE1	ACACATCACCTCCTTGTCAGACT	
	TBE-probe-WT	FAM-TGAGCCACCATCACCCAGACACA-TAMRA	
TBEV, E gene	283F1	GAGAYCAGAGTGAYCGAGGCTGG	(159)
	827R1	AGGTGGTACTTGGTTCCMTCAAGT	
	349F2	GTCAAGGCGKCTTGTGAGGCAA	
	814R2	TTCCMTCAATGTGYGCCACAGG	
A. phagocytophilum, msp2	ApMSP2f	ATGGAAGGTAGTGTTGGTTATGGTATT	(160)
	ApMSP2r	TTGGTCTTGAAGCGCTCGTA	
	ApMSP2-probe	FAM-TGGTGCCAGGGTTGAGCTTGAGATTG-TAMRA	
Ixodes spp., 16S rRNA	16Sa	CGCCTGTTTATCAAAAACAT	(161)
	16Sb	CTCCGGTTTGAACTCAGATC	

3.5. Phylogenetic analysis

BioEdit Sequence Alignment Editor 7.0.9.0 software (162) was used for the alignment and analysis of the sequences obtained in the study as well as retrieved from GenBank database. Phylogenetic analysis was performed with TreePuzzle v.5.2 and MEGA 5.0 package programs (163). The phylogenetic trees of *B. miyamotoi* partial 16S rRNA, *p66* and *glpQ* gene sequences were reconstructed using Maximum Likelihood model with 10 000 puzzling steps. The GTP model of substitution was applied for 16S rRNA partial gene (1106 bp) and Hasegawa-Kishino-Yano (HKY) model for the partial *p66* (349–355 bp) and *glpQ* (379 bp) genes. For the construction of 5S-23S IGS phylogenetic tree, the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method was used with bootstrap analysis of 1000 replicates.

3.6. Statistical analysis

For statistical analysis the confidence interval (CI) of a proportion as well as statistical significance (P) of the estimated results were calculated. The 95% CI of a proportion was estimated without a correction for continuity (164, 165). Statistical significance (P) of the *B. burgdorferi* s.l. prevalence among independent values such as tick species, stages and sites, was calculated using Fisher´s exact test 2 x 2 contingency table (166) and Poisson probability test.

4. RESULTS AND DISCUSSION

4.1. Tick-borne pathogens detected in ticks removed from birds (Paper I)

Estonia lies in the breeding area of long-distance migratory passerines, such as *Acrocephalus* spp., whose southward migration route lies from Estonia and Fennoscandia to Africa. Short-distance and partial migrants, such as *Turdus* or *Parus* species, respectively, also breed in Estonia and migrate at fall to Central and Western Europe. It has been proposed that birds, especially migrants, may play a potential role in the dispersal of tick-borne pathogens, as they may harbor infected ticks and thus introduce the infectious agents to the new natural foci (167). Overall, 249 individual ticks were removed from 178 birds of 24 species, belonging to the order Passeriformes. The majority of the birds comprised the marsh warbler (*Acrocephalus palustris*), the Eurasian reed-warbler (*A. scirpaceus*) ant the great tit (*P. major*), that accounted 34.3%, 14% and 7.9% of all birds, respectively. Most of the ticks were collected from *Acrocephalus* species (58%), followed by *Turdus* (13%), *Sylvia* (8%) and *Parus* (6%) bird species. Totally, tick-borne pathogens were detected in 13 ticks (5.2%) removed from 7 birds: one (0.4%) TBEV-infected nymph removed from marsh warbler, 11 (4.4%) ticks removed from two great tits and three common blackbirds (*Turdus merula*) were infected with *B. burgdorferi* s.l. and one (0.4%) nymph, removed from redwing (*T. iliacus*), was positive for *A. phagocytophilum* (Table 4).

Table 4. Tick-borne pathogens detected in ticks removed from migratory passerines during postbreeding migration period in 2006–2009

Bird species	Infested tick species	No. of nymphs infected / No. of nymphs on bird	Infectious agent
Marsh warbler *(A. palustris)*	*I. ricinus*	1N/4N	TBEV-Eu
Great tit *(P. major)*	*I. ricinus*	2N/2N	*B. afzelii*
Great tit *(P. major)*	*I. ricinus*	1N/1N	*B. afzelii*
Common blackbird *(T. merula)*	*I. ricinus*	4N/5N	*B. garinii*
Common blackbird *(T. merula)*	*I. ricinus*	3N/3N	*B. garinii*
Common blackbird *(T. merula)*	*I. ricinus*	1N/1N	*B. valaisiana*
Redwing *(T. iliacus)*	*I. ricinus*	1N/1N	*A. phagocytophilum*

4.1.1. TBEV

The detection of a TBEV-infected *I. ricinus* nymph removed from marsh warbler (*A. palustris*) may indicate a possible involvement of this bird species in the intercontinental distribution of TBEV. It has been shown in previous studies from Sweden (168) that tree pipits (*Anthus trivialis*), European robins (*Erithacus rubecula*), common redstarts (*Phoenicurus phoenicurus*) and song thrushes (*T. philomeros*) may contribute to a dispersal of TBEV, as ticks removed from these birds were found to be infected with TBEV with overall prevalence of 0.5%, the prevalence similar to that estimated in the current study (0.4%). The fact that TBEV, detected in *I. ricinus* from the marsh warbler in this study, belonged to the European subtype and its E gene partial sequence was closely related to the TBEV strain Korppoo-259, reported from Finland (169), could be defined by the marsh warbler's southward migration route from Finland through Estonia to South Africa. However, as one nymph out of four, feeding on the same bird, had been found to be infected with TBEV, it seems that no transmission of TBEV via co-feeding occurred on this host.

4.1.2. *Borrelia burgdorferi* s.l.

The presence of *B. burgdorferi* s.l. in bird-feeding ticks has been shown in studies from Norway, Sweden and Central Europe. However, prevalence rates vary considerably from 5–19% in Norway and Sweden (99, 170, 171) to 30–35% in Czech Republic, Switzerland and Germany (172-174). The prevalence of *B. burgdorferi* s.l., which in the current study amounted to 4.4%, was similar to those obtained in Lista Bird Observatory in southern Norway – 5.4% to 7.5%, as reported by Kjelland et al. (99).

The results of the study also support the assumption that *Turdus* spp. birds may play a crucial role in the dispersal of *B. burgdorferi* s.l. in Europe, as 8 of 11 ticks infected with *B. burgdorferi* s.l. were removed from *T. merula*. Moreover, it has been shown that common blackbirds and thrushes constitute a key avian reservoir for *B. garinii* and *B. valaisiana* in Europe (112, 121). That might also be confirmed by the results of this study as these *B. burgdorferi* s.l. genospecies were detected in 8 out of 9 ticks removed from three common blackbirds. According to the analysis of 5S-23S IGS the sequences detected in the present study were identical to those of *B. garinii* 20047, *B. garinii* NE11 and *B. valaisiana* I-214, which are also widely spread in Europe (156, 175, 176).

Borrelia afzelii is regarded as a rodent-associated genospecies for which birds are not adequate reservoirs, and moreover, the uptake of avian blood leads to the elimination of *B. afzelii* from the tick midgut (102, 177). However, the detection of *B. afzelii* in bird-feeding nymphs and even larvae has been reported in several studies from Europe (112, 170, 178, 179). As in this study *B. afzelii* was detected in nymphs, it might be suggested that it was acquired by ticks

during their previous bloodmeal on rodents as larvae. However, it is interesting that according to the analysis of 5S-23S IGS sequences of those *B. afzelii* samples, two nymphs, removed from the same great tit, had different *B. afzelii* strain combination: one nymph had only *B. afzelii* strain PGau, whereas in another nymph a mix of *B. afzelii* strains PGau and Tom1503 had been detected. This finding may indicate a transmission of *B. afzelii* PGau from one nymph to another by co-feeding. However, it is also theoretically possible, although with a very low probability, that one nymph acquired a mix of two *B. afzelii* strains at larval stage from a rodent and then eventually became feeding on the same avian host with another *B. afzelii* PGau infected nymph.

4.1.3. *Anaplasma phagocytophilum*

A previous study of Katargina et al. (180) has shown a presence of *A. phagocytophilum* in questing *I. ricinus* ticks collected from the area near Kabli and Pulgoja bird stations (site Kilingi-Nõmme) at a prevalence rate of 0.9%. It appeared to be very similar to the results of this study: only 0.4% of bird-feeding ticks were infected with *A. phagocytophilum*. A single nymph, which was infected with this tick-borne pathogen, was removed from a short-distance migrant of *Turdus* spp., the redwing (*T. iliacus*), that therefore might be considered as a potential dispenser of *A. phagocytophilum* infected ticks along its migration route between Fennoscandia and Central Europe.

4.2. Relapsing fever spirochete *Borrelia miyamotoi* in questing ticks (Paper II)

Borrelia miyamotoi was reported for the first time in 1995 by Fukunaga et al. in Japan (23), and since then it has been found in ticks in different countries in Europe. Recently it has been shown that this *Borrelia* genospecies is of medical importance, as human cases had been registered in Russia and in the USA (29-32). *Borrelia miyamotoi* from human samples in Russia belongs to the *I. persulcatus*-associated Asian type, which was also detected, for the first time in Estonia, in questing ticks of both species in the current study.

4.2.1. The prevalence of *B. miyamotoi* in questing ticks

This is the first study to report the presence of relapsing-fever group spirochete *Borrelia miyamotoi* in Estonian ticks population. Of the total 2622 ticks analyzed (2061 *I. ricinus* and 561 *I. persulcatus*), *B. miyamotoi* was detected in 23 (0.9%). The highest infection rates were observed in ticks collected in Valgamaa (2.8%), Tartumaa (1.9%) and Võrumaa (1.4%) counties (Table 5). There was no *B. miyamotoi* infection detected in ticks collected from Harjumaa, Ida-Virumaa, Viljandimaa and Pärnumaa counties. Comparing to *B. burgdorferi* s.l. complex, the infection rates of *B. miyamotoi* in ticks are

significantly lower (Paper III) (22, 24, 181, 182). It should be noted that *I. persulcatus* ticks showed significantly higher infection rates than *I. ricinus*: 15/561 (2.7%) and 8/2061 (0.4%), respectively. These values are similar to those obtained from the Moscow region, where *I. ricinus* and *I. persulcatus* are also co-distributed (29), and also to the values from Germany (182) and the USA (26, 27). As in the case of *B. burgdorferi* s.l., in the areas where the distribution ranges of *I. ricinus* and *I. persulcatus* overlap, the latter tick species shows higher infection rates. That may indicate a better vector efficiency than that of *I. ricinus* (Paper III) (83).

Table 5. *Borrelia miyamotoi* prevalence in *I. ricinus* and *I. persulcatus* ticks

County*	*IR*			*IP*			Overall % (∑overall)	
	% (∑A)	% (∑Ny)	% (∑total)	% (∑A)	% (∑Ny)	% (∑total)		
Tartumaa	0 (226)	3.0 (66)	0.7 (292)	2.7 (187)	4.3 (94)	3.2 (281)	1.9% (573)	*IP* and *IR* area 1.3% (1324**) §
Valgamaa	0 (57)	–	0 (57)	4.1 (121)	–	4.1 (121)	2.8% (178)	
Võrumaa	0 (64)	–	0 (64)	11.1 (9)	–	11.1 (9)	1.4% (73)	
Läänemaa	0 (97)	10 (10)	0.9 (107)	–	–	–	0.9% (107)	*IR* area 0.5% (1298**)§
Saaremaa	0.8 (508)	0.3 (389)	0.6 (897)	–	–	–	0.6% (897)	
Total**	0.3 (1394)	0.6 (667)	0.4 (2061) ‡	2.4 (456)	3.8 (104)	2.7 (561) ‡	0.9% (2622)	

* Only counties where *B. miyamotoi* in ticks was detected.
** The data of counties, where there was no *B. miyamotoi* detected are included.
– Not tested, *IR – I. ricinus, IP – I. persulcatus,* A – adults, Ny – nymphs.
‡ P<0.0001 Fisher's exact and Poisson probability tests.
§ P<0.05 Fisher's exact test; P<0.001 Poisson probability test.

The current study also shows statistically significant differences in infection rates of ticks in the *I. ricinus* area compared to that from the sympatric area where *I. ricinus* co-distributes with *I. persulcatus*. The total prevalence of *B. miyamotoi* in ticks collected in the sympartic areas in the eastern Estonia are almost three times as high as in ticks collected in northern and western parts of Estonia, which are allopatric for *I. ricinus* ticks only: 1.3% and 0.5%, respectively. One of the explanations may be the higher acarotropism of *B. miyamotoi* to *I. persulcatus* ticks, as it was shown for *B. burgdorferi* s.l. (83). However, *B. miyamotoi* was simultaneously detected in both tick species within the area of their co-distribution, albeit less frequently in *I. ricinus*. Thus, it might be suggested that more favorable conditions (environmental, climatic, biotic and abiotic factors) for *B. miyamotoi* circulation in eastern Estonia play more significant role. The similar findings were also demonstrated in the case of

B. burgdorferi s.l., where the prevalence rates in ticks from *I. ricinus* allopatric area were lower compared to those from sympatric area (Paper III).

The current study did not reveal any significant differences between *B. miyamotoi* infection rates in nymphs (1.0%) and adult ticks (0.8%). These data correspond to those obtained for *I. pacificus* and *I. scapularis* ticks in America as well as for *I. ricinus* in Europe (22, 26, 182). Although, the cumulative risk of *B. miyamotoi* in each developmental stage cannot be properly assessed, as no larvae were tested.

The study of Davis and Bent (183) reports that in America *B. miyamotoi* and *B. burgdorferi* circulate among different animal hosts and use different transmission loops: *B. burgdorferi* is almost exclusively transmitted to larvae that fed on a host previously infected by a nymph while *B. miyamotoi* may utilize both vertical and horizontal transmission. These observations are in correspondence with the studies from France, Germany and the USA (22, 27, 182) where no co-infections of *B. miyamotoi* with *B. burgdorferi* s.l. were found. However, the results of the present study show that in Estonia relapsing fever group spirochete *B. miyamotoi* circulates in the same environmental and climatic conditions with Lyme disease spirochete *B. burgdorferi* s.l., and that both pathogens may share hosts. It was found that 21.7% of ticks, infected with *B. miyamotoi*, were also co-infected with *B. burgdorferi* s.l. genospecies: bird-associated *B. garinii* and *B. valaisiana*, and rodent-associated *B. afzelii.* The similar finding of *B. miyamotoi* co-infection with Lyme disease spirochetes *B. afzelii* and *B. garinii* was demonstrated in *I. persulcatus* in Siberia (181). These facts may lead to a suggestion that co-infections of relapsing fever and Lyme disease spirochetes, as a result of ticks' co-feeding on the same host, depend on local climatic and environmental conditions and may occur in Estonia. Also, the single case of double infection with *B. miyamotoi* and European subtype of TBEV was demonstrated in *I. ricinus* tick from Saaremaa island that shows the co-circulations of these viral and bacterial tick-borne pathogens in natural foci as well as possible host sharing. Such double infections may also result in double infection in humans.

4.2.2. Genetic characterization of *B. miyamotoi*

The genetic characterization of Estonian samples of *B. miyamotoi* is based on sequences of three genomic regions, partial 16S rRNA (1256 bp), *p66* (532 bp) and *glpQ* (379 bp) genes. Of 23 samples, positive for *B. miyamotoi*, fourteen were amplified for all three genes, six for two genes and three samples for one gene region. Phylogenetic analysis of nucleotide sequences of these three regions showed that Estonian samples were divided into two groups, European and Asian (Figure 10). Sequences belonging to European group are identical to those amplified from *I. ricinus* from Sweden, Poland, France and the European part of Russia. Sequences within Asian group are identical to those found in

I. persulcatus, rodents and human blood in the European part of Russia, Ural, Siberia and Japan. The results of the present study demonstrated that strains of Asian type of *B. miyamotoi* may be exchanged between tick species in the sympatric area albeit at a low rate: among 16 Estonian sequences in the Asian type group only two were amplified from *I. ricinus* and 14 from *I. persulcatus* (Table 6). The similar findings of exchanging between tick species have been demonstrated in our studies on TBEV: Siberian subtype of TBEV was detected not only in its natural vector*, I. persulcatus*, but also in *I. ricinus* ticks, collected in the area of both tick species co-distribution (1).

Table 6. *Borrelia miyamotoi* infections and co-infections in *I. ricinus* and *I. persulcatus*

County	Sample	Tick species*	Sex/ stage**	*B. miyamotoi* type	Co-infection
Tartumaa	Est1868	*IP*	F	Asian	
	Est1885	*IP*	F	Asian	
	Est3943-4	*IP*	F	Asian	
	Est1811	*IP*	N	Asian	
	Est3486-4	*IP*	F	Asian	
	Est3487-4	*IP*	N	Asian	
	Est3698-2	*IP*	N	Asian	
	Est722-2	*IP*	N	Asian	
	Est1586	*IP*	N	Asian	*B. valaisiana*
	Est3115-1	*IR*	N	Asian	
	Est3489-2	*IR*	F	Asian	
Valgamaa	Est4318	*IP*	M	Asian	
	Est4350	*IP*	F	Asian	
	Est4372	*IP*	M	Asian	*B. afzelii* (VS461 group)
	Est4243	*IP*	F	Asian	
	Est4412	*IP*	F	Asian	*B. garinii* (NT29 group)
Võrumaa	Est3633	*IP*	M	Asian	*B. afzelii* (VS461 group)
Saaremaa	Est2519	*IR*	F	European	
	Est3849	*IR*	M	European	
	Est2270	*IR*	M	European	TBEV-Eu subtype
	Est2409	*IR*	M	European	*B. garinii* (20047 group)
	Est2325-3	*IR*	N	European	
Läänemaa	Est1129-4	*IR*	N	European	

**IP – Ixodes persulcatus, IR – I. ricinus.*

** F – adult female, M – adult male, N – nymph.

Resent reports of human disease caused by *B. miyamotoi* demonstrate that pathogenic *B. miyamotoi* belong to Asian (29) and American (32) types but hitherto there are no reports of human *B. miyamotoi* infection due to European type. Therefore it is still unknown whether European type of *B. miyamotoi* may cause a disease in humans. Although to date there were no human cases of a disease due to *B. miyamotoi* reported neither in Europe nor in Estonia, the detection of *B. miyamotoi* Asian type in *I. persulcatus* and, moreover, in *I. ricinus* ticks in Estonia, may raise the probability of westward distribution of pathogenic *B. miyamotoi* via adaptation to the main European tick vector,

I. ricinus. However, further investigations need to be performed to evaluate the vector efficiency of *I. ricinus* ticks for pathogenic Asian type of *B. miyamotoi*. Also, the investigations of natural reservoir animal hosts for Asian type as well as for European type *B. miyamotoi* need to be performed in Europe and in Estonia in particular.

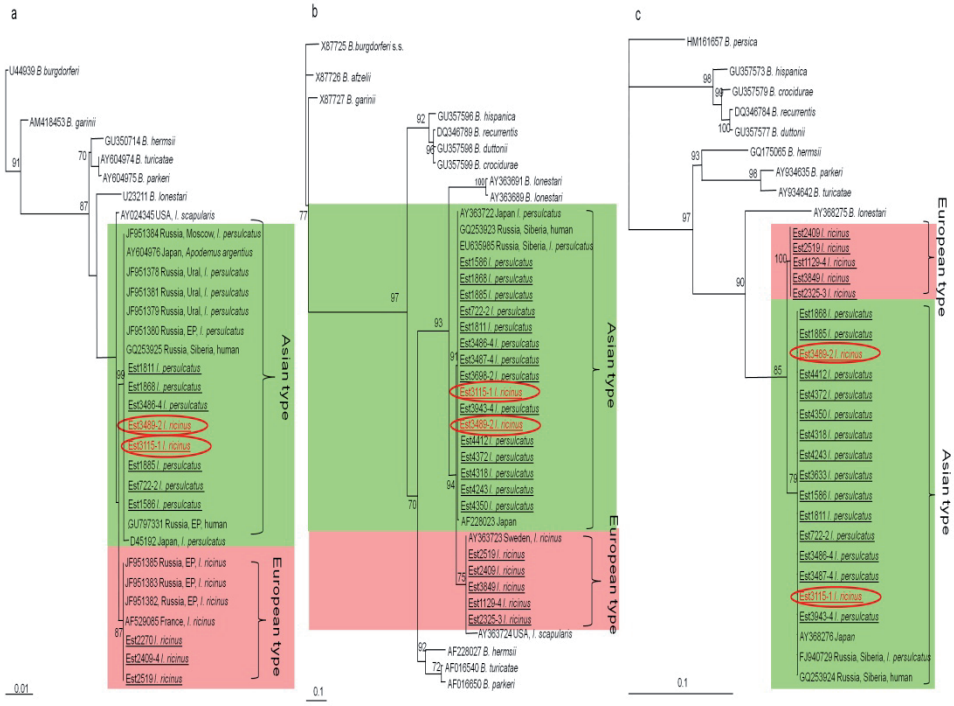

Figure 10. Phylogenetic trees based on *Borrelia miyamotoi* partial 16S rRNA, *p66* and *glpQ*. The phylogenetic trees of partial a) 16S rRNA (1106 bp), b) *p66* (349 bp and 355 bp for "*I. persulcatus*"-type and "*I. ricinus*"-type, respectively) and c) *glpQ* genes (379 bp) were constructed using Maximum Likelihood model. Quartet puzzling support values > 70% are shown. Samples sequenced in the present study are underlined. Samples of *B. miyamotoi* Asian type amplified from *I. ricinus* ticks are indicated in red ellipses.

4.3. Tick-borne borreliosis spirochete *B. burgdorferi* s.l. in questing ticks (Paper III)

The epidemiological situation of tick-borne borreliosis in Estonia is of high importance over the last decade, as the number of human cases has been drastically raised and morbidity rates remain among the highest in Europe. Thus, the evaluation of prevalence rates of *B. burgdorferi* s.l. in ticks, detection of *Borrelia* genospecies circulating in Estonia, as well as the assessment of risk areas are of national health importance.

4.3.1. The prevalence of *B. burgdorferi* s.l. in ticks from vegetation

In this study, 2833 unfed ticks, collected from the vegetation were investigated for the presence of *B. burgdorferi* s.l. Of them, 80.9% (2293/2833) belonged *to I. ricinus* species and 19.1 % (540/2833) to *I. persulcatus*.

Borrelia burgdorferi s.l. was detected in 9.7% (275/2833) of ticks, whereas *Borrelia*-positive ticks were found in all regions of study. Regionally the prevalence rates in the mainland fluctuated from 4.9% in Harjumaa to 15.6% and 17.5% in Ida-Virumaa and Valgamaa/Võrumaa, respectively. On Saaremaa, where only *I. ricinus* is found, *B. burgdorferi* s.l. was found in 10.7% of ticks (Table 7). The data obtained in the current study are similar to those reported from Sweden, Lithuania and Belarus, where overall infection rate of *B. burgdorferi* s.l. in ticks was 11%, 10.2% and 9.4%, respectively (73, 184, 185). The prevalence rates reported from Finland (5.1%) (129), although lower than infection rates demonstrated in this study for Estonia in total, are similar to those found in Harjumaa, as well as in the mainland areas where only *I. ricinus* is distributed. On the other hand, data from Latvia (also a sympatric region of *I. ricinus* and *I. persulcatus* distribution) showed prevalence as high as 25.2% that is compatible with that obtained in Valgamaa/Võrumaa.

Both tick species circulating in Estonia were found to be infected with *B. burgdorferi* s.l. However, the infection rates varied not only between *I. ricinus* and *I. persulcatus* but also depending on the area. It has been suggested that *I. persulcatus* is a more efficient vector for *B. burgdorferi* s.l. (83). The results of the current study demonstrate that the overall prevalence of *B. burgdorferi* s.l. in *I. persulcatus* ticks is twice as high as in *I. ricinus*: 16.3% and 8.6%, respectively. *Ixodes persulcatus* ticks showed also significantly higher infection rates within the area sympatric with *I. ricinus* ticks. Thus, it might be stated that in Estonia in the natural foci *I. persulcatus* ticks are more effective *B. burgdorferi* s.l. vectors, as they demonstrate higher infection rates. However, we suppose that in eastern and western parts of Estonia there might be also more favorable conditions for the *B. burgdorferi* s.l. circulation – biotic and abiotic factors, microclimate, as well as a high abundance of small and large mammals.

The prevalence rates in *I. ricinus* ticks found in allopatric area did not differ much from those in sympatric area: 8.6% and 7.4%, respectively. The differences in *B. burgdorferi* s.l. prevalence in *I. ricinus* were found within its allopatric area: *I. ricinus* ticks from Saaremaa showed double infection rate (10.7%) comparing to *I. ricinus* from mainland (5.3%). Also, previous reports from Vormsi Island (129) demonstrated 15% prevalence of *B. burgdorferi* s.l. that is similar to that found in the current study on Saaremaa Island (10.7%) and is significantly different from prevalence rate in *I. ricinus* ticks on mainland Estonia. The higher infection rate in ticks on Saaremaa is in correspondence with Lyme borreliosis morbidity, as the annual incidence rate of LB on Saaremaa has

been the highest in Estonia during the last years. Also, the recently presented ticks' distribution model based on temperature, climate and vegetation data has shown that the western coastline of Estonia as well as islands have a different ticks' distribution compared to that on the mainland (186). However, there might be other additional factors enabling more favorable conditions for pathogen circulation on Saaremaa, such as environment, mammal abundance, microclimate etc.

The current study has shown that adult ticks have higher infection rates than nymphs. Among *I. ricinus* 9.4% of adult ticks and 6.6% of nymphs had *B. burgdorferi* s.l. infection; the prevalence in *I. persulcatus* adults was 18.5% and in nymphs – 8.0% (Table 7). These findings are in correspondence with data presented in studies from Latvia (187), Finland (129) and Sweden (73) as well as with overall European values from regions with low infection rates, presented in metaanalysis (131). The higher prevalence of *B. burgdorferi* s.l. in adult ticks may also be explained by the fact that adult ticks have fed twice, as larvae and as nymphs, and thus might acquire the infection from two hosts. However, adult ticks are considered as non-significant in B. *burgdorferi* s.l. infection cycle, as adults commonly do not transmit the infection to the offspring. On the other hand, humans are often exposed to adult females that may transmit the disease-causing infection.

Table 7. *Borrelia burgdorferi* s.l. prevalence in questing ticks

County	*I. ricinus*			*I. persulcatus*			Overall % (total No.of ticks tested)
	% (No. of nymphs tested)	% (No. of adults tested)	% (∑ tested)	% (No. of nymphs tested)	% (No. of adults tested)	% (∑ tested)	
Ida-Virumaa.	9.5 (21)	4.0 (50)	5.6 (71)	10.0 (10)	23.2 (99)	22.0 (109)	15.6 (180)
Tartumaa	3.0 (67)	10.9 (230)	9.1 (297)	7.8 (102)	10.4 (192)	9.5 (294)	9.3 (591)
Võrumaa-Valgamaa	–	9.9 (121)	9.9 (121)	–	24.6 (130)	24.6 (130)	17.5 (251)
Pärnumaa	3.5 (198)	7.8 (193)	5.6 (391)	0/1[a]	4/6[a]	4/7[a]	6.5 (398)
∑sympatric area	***3.8 (286)***	***9.1 (594)***	***7.4 (880) ¥***	***7.9 (113)***	***18.5 (427)***	***16.3 (540)¥***	***10.8 (1420)*****
Harjumaa	3.4 (178)	6.6 (166)	4.9 (344)	–	–	–	4.9 (344)
Läänemaa	8.0 (100)	4.0 (100)	6.0 (200)	–	–	–	6.0 (200)
∑allopatric area, mainland	***5.0 (278)***	***5.6 (266)***	***5.3 (544) ‡‡***				***5.3 (544)*****
Saaremaa	9.4 (425)	11.9 (444)	10.7 (869) ‡‡	–	–	–	10.7 (869)
∑ allopatric area	***7.7 (703)***	***9.6 (710)***	***8.6 (1413)***				***8.6 (1413)***
∑ overall	6.6 (989) ¥¥	9.4 (1304) ¥¥,‡	8.2 (2293) *	8.0 (113) ††	18.5 (427) ††,‡	16.3 (540) *	9.7 (2833)

¥,*, ‡ , **, ‡‡ Fisher's exact and Poisson probability tests $P < 0.0001$.
a – No. of the infected/ tested; prevalence per cent was omitted due to the small number of samples.

4.3.2. *B. burgdorferi* s.l. genospecies

All 275 tick samples, positive for *B. burgdorferi* s.l., were sequenced for both partial 16S rRNA and 5S-23S IGS genes. Of them, 37 samples (13.5%) contained a mix of different *B. burgdorferi* genospecies that could not be identified individually. There were five *B. burgdorferi* s.l. genospecies identified: *B. afzelii, B. garinii, B. valaisiana, B. burgdorferi s.s.* and *B. bavariensis*. Four of these five genospecies are known to be pathogenic to humans, except *B. valaisiana* which is considered as potentially pathogenic. Up to this study, there were only few reports about detection of *B. afzelii* and *B. garinii* in ticks in Estonia (129, 130).

This study revealed that the majority of Estonian ticks, infected with *Borrelia*, were infected with *B. afzelii*, a rodent-associated genospecies: it was detected in 53.5% of all *B. burgdorferi* s.l. positive ticks, 56.1% of *I. ricinus* and 47.7% of *I. persulcatus* (Table 8). Another most frequently detected genospecies of *B. burgdorferi* s.l. group was an avian-associated *B. garinii*: 26.2% of all *B. burgdorferi* s.l. positive ticks were infected with *B. garinii*, 20.3% of *I.ricinus* and 38.6% of *I.persulcatus*. *Borrelia afzelii* and *B. garinii* are known to be most widely spread in ticks in Europe. The prevalence of these *Borrelia* genospecies obtained in the current study is similar to those reported from neighboring Latvia (187), Southern Finland (188) and Moscow region in Russia (189).

Table 8. *Borrelia burgdorferi* s.l. genotypes in *I. ricinus* and *I. persulcatus*

	I. ricinus	*I. persulcatus*	TOTAL
	No. of ticks positive/total positive (prevalence, %)	No. of ticks positive/total positive (prevalence, %)	No. of ticks positive/total positive (prevalence, %)
B. afzelii VS461	60/187 (32.1%)	32/88 (36.4%)	92/275 (33.5%)
B. afzelii NT28	37/187 (18.8%)	5/88 (5.7%)	42/275 (15.3%)
VS461+ NT28	8/187 (4.3%)	5/88 (5.7%)	13/275 (4.7%)
Total *B. afzelii*	105/187 (56.1%)	42/88 (47.7%)	147/275 (53.5%)
B. garinii 20047	38/187 (20.3%)	9/88 (10.2%)	47/275 (17.1%)
B. garinii NT29	0/187	25/88 (28.4%)	25/275 (9.1%)
Total *B. garinii*	38/187(20.3%)	34/88 (38.6%)	72/275 (26.2%)
B. valaisiana	13/ 187 (6.9%)	2/88 (2.3%)	15/275 (5.5%)
B. burgdorferi s.s.	3/187 (1.6%)	0/ 88	3/275 (1.1%)
B. bavariensis	1/187 (0.5%)	0/ 88	1/275 (0.4%)
Mix of several genotypes	27/187 (14.4%)	10/88 (11.4%)	37/275 (13.5%)

The results of this study indicate that there are two genomic subgroups of *B. afzelii* circulating in *I. ricinus* as well as in *I. persulcatus* – VS461 and NT28. The most prevalent was *B. afzelii* subgroup VS461, which comprised 62.6% of all *B. afzelii* positive ticks, followed by NT28 (28.6%). In 8.8% of ticks with *B. afzelii*, there was a mix of both subgroups detected. *B. afzelii* NT28 and VS461 are widely spread in Europe and in Asia and circulate in different vector species. Detection of these genomic subgroups in both tick species in Estonia indicates a successful co-circulation in the same environmental and biological niche as well as sharing vector ticks and animal hosts without limitations. The analysis of nucleotide sequences of partial 5S-23S IGS of Estonian samples showed that the samples within VS461 and NT28 share a high similarity rate (99.1%−100% and 98.3%−100%, respectively) with sequences reported from Sweden, Russia, Switzerland, Turkey, Belarus, Italy as well as from Korea, China, Taiwan and Japan. There were also five sublineages within subgroup NT28 and three within subgroup VS461, as revealed in this study by phylogenetic analysis of 5S-23S IGS. Thus, the results of this study are in correspondence with previous reports of a genetic variability of *B. afzelii* in Europe (Figure 11).

A recent study has shown that two genomic subgroups of *B. garinii* circulate in ticks in Estonia: 20047 ("European") and NT29 ("Asian"). Subgroup 20047, known to be circulating in both *I. ricinus* and *I. persulcatus* ticks, was the most prevalent in this study: it was detected in 65.3% of all *B. garinii*-infected ticks, however, with significant differences between *I. ricinus* (52.8%) and *I. persulcatus* (12.5%). *Borrelia garinii* subgroup NT29 was detected in 34.7% of ticks infected with *B. garinii* (Table 9). In the present study this genomic subgroup was detected only in *I. persulcatus* that clearly corresponds to the notion that NT29 has a limited geographical distribution area as it utilizes *I. persulcatus* as vector and to date has never been detected in *I. ricinus*. As *B. garinii* NT29 was also detected in *I. persulcatus* ticks in Latvia, the region of southeastern Estonia and northern and western Latvia is a unique area in Eastern Europe to study this *B. garinii* subgroup and its features that might occur due to co-distribution of two vector species. Estonian 5S-23S IGS sequences of *B. garinii* shared a high rate of similarity within both subgroups, 20047 (96.8%−100%) and NT29 (98.9%−100%), and clustered with sequences from European countries as well as from Russia and China. The phylogenetic analysis of partial 5S-23S IGS region of Estonian *B. garinii* sequences revealed five lineages within subgroup NT29 and seven lineages within subgroup 20047 (Figure 11). These results also support the notion of a wide geographic distribution of *B. garinii* as well as its genetic heterogeneity.

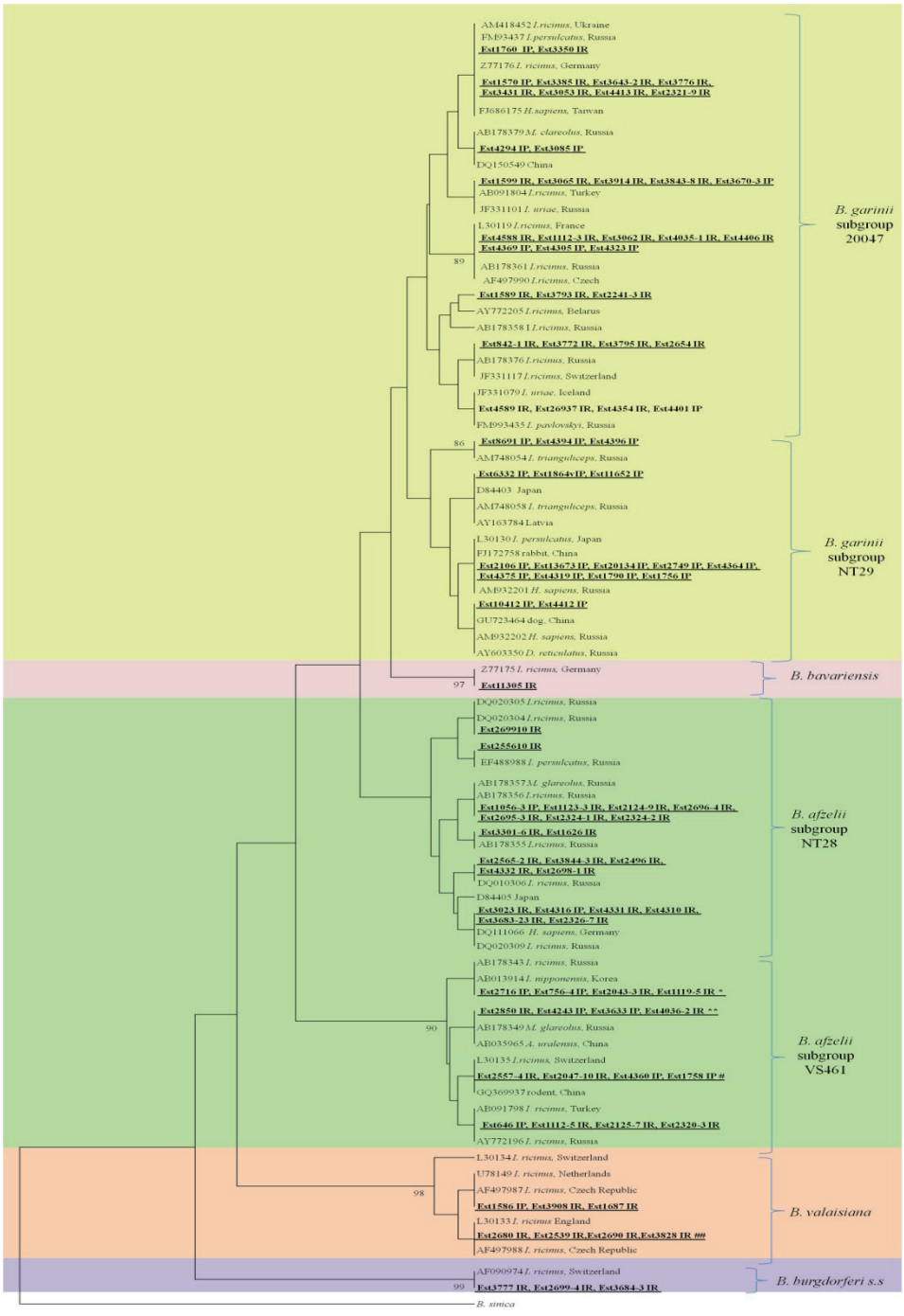

Figure 11. Phylogenetic tree of *Borrelia burgdorferi* s.l. genospecies based on 5S-23S intergenic spacer region gene.

Table 9. *Borrelia afzelii* and *B. garinii* in Estonian ticks

	I. ricinus	*I. persulcatus*	Total
B. afzelii (No.147)			
VS461	60/147 (40.8%)*	32/147 (21.8%)*	92/147 (62.6%)
NT28	37/147 (25.2%)**	5/147 (3.4%)**	42/147 (28.6%)
VS461/NT28 mix	8/147 (5.4%)	5/147 (3.4%)	13/147 (8.8%)
B. garinii (No. 72)			
20047	38/72 (52.8%)†	9/72 (12.5%)†	47/72 (65.3%)
NT29	0/72	25/72 (34.7%)	25/72 (34.7%)

*, **, † Fisher's exact test P<0.001.

Another avian-associated *B. burgdorferi* s.l. genospecies, detected in this study, was *B. valaisiana*, which was found in 5.5% of ticks. The similar infection rates were also reported from Sweden and Norway. Interestingly, *B. valaisiana* was detected in both *I. ricinus* and *I. persulcatus* (Table 8), albeit the detection of this *Borrelia* genospecies in *I. persulcatus* was rarely reported previously. It might be proposed, that in the areas of I. *ricinus* and *I. persulcatus* co-distribution, *B. valaisiana* may exchange vectors and effectively utilize both tick species: similarly to this study it was also detected in *I. persulcatus* in sympatric areas in Latvia and Baltic regions of Russia (190, 191). The similar vector species exchange has also been shown for TBEV in Estonia and in Latvia (1) as well as for *B. miyamotoi* (Paper II). By such switching to other tick species tick-borne pathogens may adapt to some new vectors that may result in the spreading of these pathogens onto new areas. The phylogenetic analysis of 5S-23S IGS sequences showed that Estonian samples of *B. valaisiana* cluster with high similarity rates (98.8%−100%) with sequences from Czech Republic, UK and Netherlands and belong to *B. valaisiana* genomic group VS116.

This study has also shown the presence of *B. burgdorferi* s.s. for the first time in Estonia, although at a low rate: it was detected in 0.1% of all ticks analyzed and in 1.6% of *Borrelia*-positive *I. ricinus* ticks (Table 8). Similarly, low infection rates, from 0.3% to 2.1%, for this *Borrelia* genospecies have also been reported from Latvia (187), Belarus (185) and Baltic regions of Russia (190) as well as from Finland (129) and Sweden (73). However, the data presented in metaanalysis reported about 16% of ticks to be infected with *B. burgdorferi* s.s. in a region of Norway, Sweden, Finland and Estonia (131). This *Borrelia* genospecies was found only in *I. ricinus* ticks, collected from Tartumaa and Saaremaa island, suggesting that it utilizes only this tick species as vector. This notion can also be supported by the fact that to date *B. burgdorferi* s.s. has never been reported from areas, where *I. persulcatus* is sympatric with *I. ricinus* or dominating. An analysis of Estonian sequences of *B. burgdorferi* s.s. has shown that they are identical to each other and cluster with those detected in ticks and

rodents in the USA and Europe as well as in humans in Czech Republic (Figure 11).

Another *B. burgdorferi* s.l. genospecies detected in Estonian tick populations for the first time was *B. bavariensis*, which was found in one *I. ricinus* tick from Läänemaa that comprised 0.4% of all *B. burgdorferi* s.l.-positive ticks (Table 8). The nucleotide sequence of 5S-23S IGS of Estonian sample was identical to the strain PBi from Germany, which is a prototype strain for *B. bavariensis*. It has been suggested that *B. bavariensis* is restricted to Central Europe (192) – Switzerland (176, 193), Germany, Austria and Czech Republic (194) – and thus, associated with *I. ricinus* as a vector, that was also in correspondence with detecting of this *Borrelia* genospecies in *I. ricinus* in this study.

CONCLUSIONS

- Three medically important tick-borne pathogens were found in *I. ricinus* ticks removed from short-distance as well as long-distance migratory birds: European subtype of tick-borne encephalitis virus, Lyme borreliosis causing spirochetes *B. garinii*, *B. valaisiana* and *B. afzelii,* and *Anaplasma phagocytophilum*, the causative agent of human anaplasmosis. The detection of *B. garinii* and *B. valaisiana* in *I. ricinus* nymphs removed from birds of *Turdus* sp. may indicate the acquiring of these infections from birds. Blackbirds and thrushes are known as reservoir hosts for *B. garinii* and *B. valaisiana*. However, the detection of a combination of not avian-associated *B. afzelii* strains in nymphs, removed from a great tit may indicate acquisition of infectious agent by co-feeding. The detection of TBEV-Eu in a tick removed from a long-distance migrant marsh warbler may indicate a potential role of this avian species in dissemination of infected ticks over long distance and introducing TBPs to new foci. Overall, the results of this study support the notion that migratory avian species may play important role in the dissemination of ticks and tick-borne pathogens through the migration route from Fennoscandia to Central Europe and Africa.
- The presence in Estonian tick populations of pathogenic relapsing fever spirochete *B. miyamotoi* was shown for the first time. *Ixodes persulcatus* showed significantly higher infection rates and therefore the higher acarotropism of *B. miyamotoi* to *I. persulcatus* was presumed. The detection of *I. persulcatus*-associated Asian type *B. miyamotoi* in *I. ricinus* may indicate a vector exchange possibility and thus, potential introducing of pathogenic *B. miyamotoi* Asian type into the distribution range of *I. ricinus*. Co-infections with *B. burgdorferi* s.l. demonstrate sharing hosts in the same foci. The phylogenetic analysis showed that Estonian sequences within European type of *Borrelia miyamotoi* are closely related to strains circulating in European countries, whereas those within Asian type are related to strains circulating in Russia and Asia and found in ticks and human patients.
- Five *Borrelia burgdorferi* s.l. genospecies were detected in Estonian tick populations for the first time, of which four are pathogenic: B. *afzelii, B. garinii, B. bavariensis* and *B. burgdorferi* s.s. The most prevalent, *B. afzelii* and *B. garinii*, represent the main causative agents of Lyme borreliosis in Europe. Avian-associated and potentially pathogenic *B. valaisiana* was detected mostly in *I. ricinus* but also in *I. persulcatus*, that is a rare event. Phylogenetic analysis demonstrated that Estonian sequences of *B. burgdorferi* s.l genospecies are closely related to those detected in tick, animal and human samples and circulating in European and Asian countries as well as in Russia.

- The highest infection rates of ticks with *Borrelia* genospecies were demonstrated for the southern and eastern parts of Estonia (Valgamaa, Tartumaa, Võrumaa and Ida-Virumaa), which are sympatric areas of *I. ricinus* and *I. persulcatus* co-distribution, as well as for Saaremaa and Muhu islands.

REFERENCES

1. Katargina O, Russakova S, Geller J, Kondrusik M, Zajkowska J, Zygutiene M, et al. Detection and characterization of tick-borne encephalitis virus in Baltic countries and Eastern Poland. PLoS One. 2013; 8 (5) :e61374.
2. Hall SS. Iceman autopsy. National Geographic Magazine. 2011.
3. Summerton N. Lyme disease in the eighteenth century BMJ. 1995 Dec 2; 311 (7018).
4. Buchwald A. Ein Fall von diffuser idiopathischer Haut-Atrophie. Arch Dermatol Syph. 1883; 15: 553-6.
5. Marcus K. Verhandlungen der dermatologischen Gesellschaft zu Stockholm. Archiv für Dermatologie und Syphilis. 1910; 101 (2-3): 403-6.
6. Afzelius A. Erythema migrans. Acta Derm Venereol. 1921; 2: 120-5.
7. Hellerström S. Erythema chronicum migrans Afzelii. Archiv Dermatologie and Venereologie. 1930; 11: 315-21.
8. Hellerström S. Erythema chronicum migrans Afzelius with meningitis. Acta Derm Venereol. 1951 (31): 227-34.
9. Bianchi GE. [Penicillin therapy of lymphocytoma]. Dermatologica. 1950; 100(4-6): 270-3.
10. Thyresson N. The penicillin treatment of acrodermatitis atrophicans chronica (Herxheimer). Acta Derm Venereol. 1949; 29(6): 572-621.
11. Steere AC, Malawista SE, Snydman DR, Shope RE, Andiman WA, Ross MR, et al. Lyme arthritis: an epidemic of oligoarticular arthritis in children and adults in three Connecticut communities. Arthritis Rheum. 1977 Jan-Feb; 20(1): 7-17.
12. Steere AC, Batsford WP, Weinberg M, Alexander J, Berger HJ, Wolfson S, et al. Lyme carditis: cardiac abnormalities of Lyme disease. Ann Intern Med. 1980 Jul; 93 (1): 8-16.
13. Reik L, Steere AC, Bartenhagen NH, Shope RE, Malawista SE. Neurologic abnormalities of Lyme disease. Medicine (Baltimore). 1979 Jul; 58 (4): 281-94.
14. Steere AC, Hardin JA, Malawista SE. Lyme arthritis: a new clinical entity. Hosp Pract. 1978 Apr; 13 (4): 143-58.
15. Burgdorfer W, Barbour AG, Hayes SF, Benach JL, Grunwaldt E, Davis JP. Lyme disease-a tick-borne spirochetosis? Science. 1982 Jun 18; 216 (4552): 1317-9.
16. Stanek G, Reiter M. The expanding Lyme *Borrelia* complex – clinical significance of genomic species? Clin Microbiol Infect. 2011 Apr; 17 (4): 487 – 93.
17. Gray JS. The ecology of ticks transmitting Lyme borreliosis. Exp Appl Acarol. 1998 (22): 249-58.
18. Barbour A. *Borrelia*: a diverse and ubiquitous genus of tick-borne pathogens. In: Scheld W, Craig, WA., Hughes JM., editor. Emerging infections. Washington: American Society for Microbiology Press; 2001. p. 153-74.

19. Barbour AG, Hayes SF. Biology of *Borrelia* species. Microbiol Rev. 1986 Dec; 50 (4): 381-400.

20. Koenen F, Pascussi, I., Jaenson, T., Madder, M., de Sousa, R., Estrada-Pena, A., Farkas, R., Salman, M. Tick-borne infections (including zoonoses) in Europe and the Mediterranean basin. Ticks and tick-borne diseases: geographical distribution and control strategies in the Euro-Asia region: CABI; 2013. p. 33-54.

21. Fournier PE, Ndihokubwayo JB, Guidran J, Kelly PJ, Raoult D. Human pathogens in body and head lice. Emerg Infect Dis. 2002 Dec; 8 (12): 1515-8.

22. Barbour AG, Bunikis J, Travinsky B, Hoen AG, Diuk-Wasser MA, Fish D, et al. Niche partitioning of *Borrelia burgdorferi* and *Borrelia miyamotoi* in the same tick vector and mammalian reservoir species. Am J Trop Med Hyg. 2009 Dec; 81 (6): 1120-31.

23. Fukunaga M, Takahashi Y, Tsuruta Y, Matsushita O, Ralph D, McClelland M, et al. Genetic and phenotypic analysis of *Borrelia miyamotoi* sp. nov., isolated from the ixodid tick *Ixodes persulcatus*, the vector for Lyme disease in Japan. Int J Syst Bacteriol. 1995 Oct; 45 (4): 804-10.

24. Fomenko NV, Livanova NN, Borgoiakov V, Kozlova IV, Shulaikina IV, Pukhovskaia NM, et al. [Detection of *Borrelia miyamotoi* in ticks *Ixodes persulcatus* from Russia]. Parazitologiia. 2010 May-Jun; 44 (3): 201-11.

25. Wilhelmsson P, Fryland L, Borjesson S, Nordgren J, Bergstrom S, Ernerudh J, et al. Prevalence and diversity of *Borrelia* species in ticks that have bitten humans in Sweden. J Clin Microbiol. 2010 Nov; 48 (11): 4169-76.

26. Mun J, Eisen RJ, Eisen L, Lane RS. Detection of a *Borrelia miyamotoi* sensu lato relapsing-fever group spirochete from *Ixodes pacificus* in California. J Med Entomol. 2006 Jan; 43 (1): 120-3.

27. Scoles GA, Papero M, Beati L, Fish D. A relapsing fever group spirochete transmitted by *Ixodes scapularis* ticks. Vector Borne Zoonotic Dis. 2001 Spring; 1 (1): 21-34.

28. Ullmann AJ, Gabitzsch ES, Schulze TL, Zeidner NS, Piesman J. Three multiplex assays for detection of *Borrelia burgdorferi* sensu lato and *Borrelia miyamotoi* sensu lato in field-collected *Ixodes* nymphs in North America. J Med Entomol. 2005 Nov; 42 (6): 1057-62.

29. Platonov AE, Karan LS, Kolyasnikova NM, Makhneva NA, Toporkova MG, Maleev VV, et al. Humans infected with relapsing fever spirochete *Borrelia miyamotoi*, Russia. Emerg Infect Dis. 2012 Oct;17(10):1816-23.

30. Sarksyan DS, Platonov AE, Karan LS, Malinin IE, Khalitova LI, Shakhov VI, et al. [Clinical presentation of "new" tick-borne borreliosis caused by *Borrelia miyamotoi*]. Ter Arkh. 2012; 84 (11): 34-41.

31. Gugliotta JL, Goethert HK, Berardi VP, Telford SR, 3rd. Meningoencephalitis from *Borrelia miyamotoi* in an immunocompromised patient. N Engl J Med. 2013 Jan 17;368(3):240-5.

32. Krause PJ, Narasimhan S, Wormser GP, Rollend L, Fikrig E, Lepore T, et al. Human *Borrelia miyamotoi* infection in the United States. N Engl J Med. 2013 Jan 17; 368 (3): 291-3.

33. Taylor LH, Latham SM, Woolhouse ME. Risk factors for human disease emergence. Philos Trans R Soc Lond B Biol Sci. 2001 Jul 29; 356 (1411): 983-9.

34. Prentice MB, Rahalison L. Plague. Lancet. 2007 Apr 7; 369 (9568): 1196-207.

35. Kaiser R. Tick-borne encephalitis. Infect Dis Clin North Am. 2008 Sep; 22 (3): 561-75.

36. Dumler JS, Madigan JE, Pusterla N, Bakken JS. Ehrlichioses in humans: epidemiology, clinical presentation, diagnosis, and treatment. Clin Infect Dis. 2007 Jul 15; 45 Suppl 1: S45-51.

37. Lindgren E, Jaenson, T. Lyme borreliosis in Europe. Influences of climate and climate change, epidemiology, ecology and adaptation measures. World Health Organization; 2006.

38. CDC. Reported cases of Lyme disease by state or locality, 2002-2011, http://www.cdc.gov/Lyme/stats/chartstables/reportedcases_statelocality.html, 3. August 2013.

39. Rosa PA, Tilly K, Stewart PE. The burgeoning molecular genetics of the Lyme disease spirochaete. Nat Rev Microbiol. 2005 Feb; 3 (2): 129-43.

40. Guerau-de-Arellano M, Huber BT. Chemokines and Toll-like receptors in Lyme disease pathogenesis. Trends Mol Med. 2005 Mar; 11 (3): 114-20.

41. Hovind-Hougen K. Ultrastructure of spirochetes isolated from *Ixodes ricinus* and *Ixodes dammini*. Yale J Biol Med. 1984 Jul-Aug; 57 (4): 543-8.

42. Rosa PA. Microbiology of *Borrelia burgdorferi*. Semin Neurol. 1997 Mar; 17 (1): 5-10.

43. Caimano M. The genus *Borrelia*. The prokaryotes. 3rd ed. New York: Springer; 2006. p. 235-93.

44. Haake DA. Spirochaetal lipoproteins and pathogenesis. Microbiology. 2000 Jul; 146 (Pt 7): 1491-504.

45. Radolf JD, Caimano MJ, Stevenson B, Hu LT. Of ticks, mice and men: understanding the dual-host lifestyle of Lyme disease spirochaetes. Nat Rev Microbiol. 2012 Feb; 10 (2): 87-99.

46. Takayama K, Rothenberg RJ, Barbour AG. Absence of lipopolysaccharide in the Lyme disease spirochete, *Borrelia burgdorferi*. Infect Immun. 1987 Sep; 55 (9): 2311-3.

47. Motaleb MA, Corum L, Bono JL, Elias AF, Rosa P, Samuels DS, et al. *Borrelia burgdorferi* periplasmic flagella have both skeletal and motility functions. Proc Natl Acad Sci USA. 2000 Sep 26; 97 (20): 10899-904.

48. Casjens S, Palmer N, van Vugt R, Huang WM, Stevenson B, Rosa P, et al. 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. 2000 Feb; 35 (3): 490-516.

49. Murgia R, Cinco M. Induction of cystic forms by different stress conditions in *Borrelia burgdorferi*. APMIS. 2004 Jan; 112 (1): 57-62.

50. Gruntar I, Malovrh T, Murgia R, Cinco M. Conversion of *Borrelia garinii* cystic forms to motile spirochetes in vivo. APMIS. 2001 May; 109 (5): 383-8.

51. Casjens S, Eggers, C., Schwarts, I. *Borrelia* genomics: chromosome, plasmids, bacteriophages and genetic variation. Radolf J, Samuels S. (editors). *Borrelia*: molecular biology, host interaction and pathogenesis: Horizon Scientific Press 2010. p. 27-53.

52. Piesman J, Schwan, T. Ecology of Borreliae and their arthropod vectors. Radolf J, Samuels S., (editors). *Borrelia*: molecular biology, host interaction and pathogenesis. Horizon Scientific Press; 2010. p. 251-78.

53. Fingerle V, Schulte-Spechtel UC, Ruzic-Sabljic E, Leonhard S, Hofmann H, Weber K, et al. Epidemiological aspects and molecular characterization of *Borrelia burgdorferi* s.l. from southern Germany with special respect to the new species *Borrelia spielmanii* sp. nov. Int J Med Microbiol. 2008 Apr; 298 (3-4): 279-90.

54. Stanek G, Strle F. Lyme disease: European perspective. Infect Dis Clin North Am. 2008 Jun; 22 (2): 327-39.

55. Baranton G, Postic D, Saint Girons I, Boerlin P, Piffaretti JC, Assous M, et al. Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis. Int J Syst Bacteriol. 1992 Jul; 42 (3): 378-83.

56. Alekseev AN, Dubinina, H.V., and Jushkova, O.V. Influence of Anthropogenic Pressure on the System "Tick-tick-borne Pathogens": Pensoft Pub; 2010.

57. Canica MM, Nato F, du Merle L, Mazie JC, Baranton G, Postic D. Monoclonal antibodies for identification of *Borrelia afzelii* sp. nov. associated with late cutaneous manifestations of Lyme borreliosis. Scand J Infect Dis. 1993; 25 (4): 441-8.

58. Gorelova NB, Korenberg EI, Kovalevskii Iu V, Postic D, Baranton G. [The isolation of *Borrelia* from the tick *Ixodes trianguliceps* (Ixodidae) and the possible significance of this species in the epizootiology of ixodid tick-borne borrelioses]. Parazitologiia. 1996 Jan-Feb; 30 (1): 13-8.

59. Korenberg EI, Nefedova VV, Romanenko VN, Gorelova NB. The tick *Ixodes pavlovskyi* as a host of spirochetes pathogenic for humans and its possible role in the epizootiology and epidemiology of borrelioses. Vector Borne Zoonotic Dis. 2010 Jun; 10 (5): 453-8.

60. Olsen B, Jaenson TG, Noppa L, Bunikis J, Bergstrom S. A Lyme borreliosis cycle in seabirds and *Ixodes uriae* ticks. Nature. 1993 Mar 25; 362 (6418): 340-2.

61. Smith RP, Jr., Muzaffar SB, Lavers J, Lacombe EH, Cahill BK, Lubelczyk CB, et al. *Borrelia garinii* in seabird ticks (*Ixodes uriae*), Atlantic Coast, North America. Emerg Infect Dis. 2006 Dec; 12 (12): 1909-12.

62. Larsson C, Comstedt P, Olsen B, Bergstrom S. First record of Lyme disease *Borrelia* in the Arctic. Vector Borne Zoonotic Dis. 2007 Fall; 7 (3): 453-6.

63. Margos G, Vollmer SA, Cornet M, Garnier M, Fingerle V, Wilske B, et al. A new *Borrelia* species defined by multilocus sequence analysis of housekeeping genes. Appl Environ Microbiol. 2009 Aug; 75 (16): 5410-6.

64. Postic D, Ras NM, Lane RS, Hendson M, Baranton G. Expanded diversity among Californian *Borrelia* isolates and description of *Borrelia bissettii* sp. nov. (formerly *Borrelia* group DN127). J Clin Microbiol. 1998 Dec; 36 (12): 3497-504.

65. Richter D, Postic D, Sertour N, Livey I, Matuschka FR, Baranton G. Delineation of *Borrelia burgdorferi* sensu lato species by multilocus sequence analysis and confirmation of the delineation of *Borrelia spielmanii* sp. nov. Int J Syst Evol Microbiol. 2006 Apr; 56 (Pt 4) :873-81.

66. Richter D, Schlee DB, Allgower R, Matuschka FR. Relationships of a novel Lyme disease spirochete, *Borrelia spielmani* sp. nov., with its hosts in Central Europe. Appl Environ Microbiol. 2004 Nov; 70 (11) :6414-9.

67. Le Fleche A, Postic D, Girardet K, Peter O, Baranton G. Characterization of *Borrelia lusitaniae* sp. nov. by 16S ribosomal DNA sequence analysis. Int J Syst Bacteriol. 1997 Oct; 47 (4): 921-5.

68. Wang G, van Dam AP, Le Fleche A, Postic D, Peter O, Baranton G, et al. Genetic and phenotypic analysis of *Borrelia valaisiana* sp. nov. (*Borrelia* genomic groups VS116 and M19). Int J Syst Bacteriol. 1997 Oct; 47 (4): 926-32.

69. Masuzawa T. Terrestrial distribution of the Lyme borreliosis agent *Borrelia burgdorferi* sensu lato in East Asia. Jpn J Infect Dis. 2004 Dec; 57 (6): 229-35.

70. Takada N, Fujita H, Yano Y, Ishiguro F, Iwasaki H, Masuzawa T. First records of tick-borne pathogens, *Borrelia*, and spotted fever group Rickettsiae in Okinawajima Island, Japan. Microbiol Immunol. 2001; 45 (2): 163-5.

71. Margos G, Hojgaard A, Lane RS, Cornet M, Fingerle V, Rudenko N, et al. Multilocus sequence analysis of *Borrelia bissettii* strains from North America reveals a new *Borrelia* species, *Borrelia kurtenbachii*. Ticks Tick Borne Dis. 2010 Dec; 1 (4): 151-8.

72. Hamer SA, Hickling GJ, Keith R, Sidge JL, Walker ED, Tsao JI. Associations of passerine birds, rabbits, and ticks with *Borrelia miyamotoi* and *Borrelia andersonii* in Michigan, USA. Parasit Vectors. 2012; 5: 231.

73. Fraenkel CJ, Garpmo U, Berglund J. Determination of novel *Borrelia* genospecies in Swedish *Ixodes ricinus* ticks. J Clin Microbiol. 2002 Sep; 40 (9): 3308-12.

74. Margos G, Castillo-Ramirez S, Hoen AG. Phylogeography of Lyme borreliosis-group spirochetes and methicillin-resistant *Staphylococcus aureus*. Parasitology. 2012 Dec; 139 (14): 1952-65.

75. Richter D, Debski A, Hubalek Z, Matuschka FR. Absence of Lyme disease spirochetes in larval *Ixodes ricinus* ticks. Vector Borne Zoonotic Dis. 2012 Jan; 12 (1): 21-7.

76. Eisen L, Lane, R.S. Vectors of *Borrelia burgdorferi* sensu lato. Kahl O, Lane, R. S., Stanek, G. (editors). Lyme borreliosis: biology, epidemiology and control. Cambridge, MA, USA: CABI; 2002. p. 91-115.

77. Balashov Iu S. Bloodsucking ticks (Ixodoidea) - vectors of diseases of man and animals. Miscellaneous Publications of the Entomological Society of America. 1972; 8: 163-376.

78. Talleklint L, Jaenson TG. Transmission of *Borrelia burgdorferi* s.l. from mammal reservoirs to the primary vector of Lyme borreliosis, *Ixodes ricinus* (Acari: Ixodidae), in Sweden. J Med Entomol. 1994 Nov; 31 (6): 880-6.

79. Randolph S, Gern L. Co-feeding transmission and its contribution to the perpetuation of the Lyme disease spirochete *Borrelia afzelii*. Emerg Infect Dis. 2003 Jul; 9 (7): 893-4; author reply 5-6.

80. Richter D, Allgower R, Matuschka FR. Co-feeding transmission and its contribution to the perpetuation of the Lyme disease spirochete *Borrelia afzelii*. Emerg Infect Dis. 2002 Dec; 8 (12): 1421-5.

81. Rollend L, Fish D, Childs JE. Transovarial transmission of *Borrelia* spirochetes by *Ixodes scapularis*: a summary of the literature and recent observations. Ticks Tick Borne Dis. 2013 Feb; 4 (1-2): 46-51.

82. Alekseev AN, Dubinuna, H.V., Jushakova, O.V. Features of sexual behavior and its importance for the maintenance of circulation of the emerging infections' pathogens. Alimov AF (editor). Functioning of the "Tick-Pathogens" parasityc system under the influence of increasing anthropogenic pressing. Saint Petersburg; 2008. p. 84-8.

83. Korenberg EI, Kovalevskii YV, Levin ML, Shchyogoleva TV. The prevalence of *Borrelia burgdorferi* sensu lato in *Ixodes persulcatus* and *I. ricinus* ticks in the zone of their sympatry. Folia Parasitol (Praha). 2001;48(1):63-8.

84. Vasilenko VA, Pototskii AA, Chernyshova MG. [Tick-borne encephalitis in the Estonian SSR]. Zh Mikrobiol Epidemiol Immunobiol. 1990 Sep (9): 43-7.

85. Swanson SJ, Neitzel D, Reed KD, Belongia EA. Coinfections acquired from *Ixodes* ticks. Clin Microbiol Rev. 2006 Oct; 19 (4): 708-27.

86. Uspensky I. Ability of successful attack in two species of ixodid ticks (Acari: Ixodidae) as a manifestation of their aggressiveness. Exp Appl Acarol. 1993 Sep; 17 (9): 673-83.

87. Gray JS. The development and seasonal activity of the tick *Ixodes ricinus*: a vector of Lyme borreliosis. Review of Medical and Veterinary Entomology. 1991 (79): 323-33.

88. Okulova NM. The vertical and horizontal movement of Ixodidae ticks in the forest in depending from the temperature and air humidity. Russian Journal of Ecology. 1978; 2: 44-8.

89. Jaenson TG, Talleklint L, Lundqvist L, Olsen B, Chirico J, Mejlon H. Geographical distribution, host associations, and vector roles of ticks (Acari: Ixodidae, Argasidae) in Sweden. J Med Entomol. 1994 Mar; 31 (2): 240-56.

90. Piesman J, Oliver JR, Sinsky RJ. Growth kinetics of the Lyme disease spirochete (*Borrelia burgdorferi*) in vector ticks (*Ixodes dammini*). Am J Trop Med Hyg. 1990 Apr; 42 (4): 352-7.

91. Meiners T, Hammer B, Gobel UB, Kahl O. Determining the tick scutal index allows assessment of tick feeding duration and estimation of infection risk with *Borrelia burgdorferi* sensu lato in a person bitten by an *Ixodes ricinus* nymph. Int J Med Microbiol. 2006 May; 296 Suppl 40: 103-7.

92. Piesman J, Mather TN, Sinsky RJ, Spielman A. Duration of tick attachment and *Borrelia burgdorferi* transmission. J Clin Microbiol. 1987 Mar; 25 (3): 557-8.

93. Schwan TG, Piesman J, Golde WT, Dolan MC, Rosa PA. Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. Proc Natl Acad Sci USA. 1995 Mar 28; 92 (7): 2909-13.

94. Pal U, Li X, Wang T, Montgomery RR, Ramamoorthi N, Desilva AM, et al. TROSPA, an *Ixodes scapularis* receptor for *Borrelia burgdorferi*. Cell. 2004 Nov 12; 119 (4): 457-68.

95. Hovius JW, van Dam AP, Fikrig E. Tick-host-pathogen interactions in Lyme borreliosis. Trends Parasitol. 2007 Sep; 23 (9): 434-8.

96. Brissette CA, Haupt K, Barthel D, Cooley AE, Bowman A, Skerka C, et al. *Borrelia burgdorferi* infection-associated surface proteins ErpP, ErpA, and ErpC bind human plasminogen. Infect Immun. 2009 Jan; 77 (1): 300-6.

97. Fuchs H, Wallich R, Simon MM, Kramer MD. The outer surface protein A of the spirochete *Borrelia burgdorferi* is a plasmin(ogen) receptor. Proc Natl Acad Sci USA. 1994 Dec 20; 91 (26): 12594-8.

98. Hu LT, Perides G, Noring R, Klempner MS. Binding of human plasminogen to *Borrelia burgdorferi*. Infect Immun. 1995 Sep; 63 (9): 3491-6.

99. Kjelland V, Stuen S, Skarpaas T, Slettan A. *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks collected from migratory birds in Southern Norway. Acta Vet Scand. 2010; 52-9.

100. Derdakova M, Lencakova D. Association of genetic variability within the *Borrelia burgdorferi* sensu lato with the ecology, epidemiology of Lyme borreliosis in Europe. Ann Agric Environ Med. 2005; 12 (2): 165-72.

101. Kurtenbach K, Schäfer S., de Michelis, S., Etti, S., Sewel, H. *Borrelia burgdorferi* sensu lato in the vertebrate host. Kahl O, Lane , R. S., Stanek, G. (editors). Lyme borreliosis: biology, epidemiology and control. Cambridge, MA, USA: CABI; 2002. p. 117-48.

102. Hanincova K, Schafer SM, Etti S, Sewell HS, Taragelova V, Ziak D, et al. Association of *Borrelia afzelii* with rodents in Europe. Parasitology. 2003 Jan; 126 (Pt 1): 11-20.

103. Pawelczyk A, Ogrzewalska M, Zadrozna I, Sinski E. The zoonotic reservoir of *Borrelia burgdorferi* sensu lato in the Mazury Lakes district of North-Eastern Poland. Int J Med Microbiol. 2004 Apr; 293 Suppl 37: 167-71.

104. Matuschka FR, Allgower R, Spielman A, Richter D. Characteristics of garden dormice that contribute to their capacity as reservoirs for Lyme disease spirochetes. Appl Environ Microbiol. 1999 Feb; 65 (2): 707-11.

105. Matuschka FR, Eiffert H, Ohlenbusch A, Spielman A. Amplifying role of edible dormice in Lyme disease transmission in Central Europe. J Infect Dis. 1994 Jul; 170 (1): 122-7.

106. Richter D, Schlee DB, Matuschka FR. Reservoir competence of various rodents for the Lyme disease spirochete *Borrelia spielmanii*. Appl Environ Microbiol. 2011 Jun; 77 (11): 3565-70.

107. Matuschka FR, Endepols S, Richter D, Spielman A. Competence of urban rats as reservoir hosts for Lyme disease spirochetes. J Med Entomol. 1997 Jul; 34 (4): 489-93.

108. Humair PF, Gern L. Relationship between *Borrelia burgdorferi* sensu lato species, red squirrels (*Sciurus vulgaris*) and *Ixodes ricinus* in enzootic areas in Switzerland. Acta Trop. 1998 Jun; 69 (3): 213-27.

109. Gray JS, Kahl O, Janetzki-Mittman C, Stein J, Guy E. Acquisition of *Borrelia burgdorferi* by *Ixodes ricinus* ticks fed on the European hedgehog, *Erinaceus europaeus L*. Exp Appl Acarol. 1994 Aug; 18 (8): 485-91.

110. Talleklint L, Jaenson TG. Maintenance by hares of European *Borrelia burgdorferi* in ecosystems without rodents. J Med Entomol. 1993 Jan; 30 (1): 273-6.

111. Grigor'eva LA. [Shrews as a *Borrelia* reservoir in northwestern Russia]. Parazitologiia. 1996 Sep-Oct; 30 (5): 458-60.

112. Taragel'ova V, Koci J, Hanincova K, Kurtenbach K, Derdakova M, Ogden NH, et al. Blackbirds and song thrushes constitute a key reservoir of *Borrelia garinii*, the causative agent of borreliosis in Central Europe. Appl Environ Microbiol. 2008 Feb; 74 (4): 1289-93.

113. Dubska L, Literak I, Kocianova E, Taragelova V, Sverakova V, Sychra O, et al. Synanthropic birds influence the distribution of *Borrelia* species: analysis of *Ixodes ricinus* ticks feeding on passerine birds. Appl Environ Microbiol. 2011 Feb; 77 (3): 1115-7.

114. Kurtenbach K, Carey D, Hoodless AN, Nuttall PA, Randolph SE. Competence of pheasants as reservoirs for Lyme disease spirochetes. J Med Entomol. 1998 Jan; 35 (1): 77-81.

115. Kjelland V, Ytrehus B, Stuen S, Skarpaas T, Slettan A. Prevalence of *Borrelia burgdorferi* in *Ixodes ricinus* ticks collected from moose (*Alces alces*) and roe deer (*Capreolus capreolus*) in Southern Norway. Ticks Tick Borne Dis. 2011 Jun; 2 (2): 99-103.

116. Ogden NH, Nuttall PA, Randolph SE. Natural Lyme disease cycles maintained via sheep by co-feeding ticks. Parasitology. 1997 Dec; 115 (Pt 6): 591-9.

117. De Boer R, Hovius KE, Nohlmans MK, Gray JS. The woodmouse (*Apodemus sylvaticus*) as a reservoir of tick-transmitted spirochetes (*Borrelia burgdorferi*) in The Netherlands. Zentralbl Bakteriol. 1993 Aug; 279 (3): 404-16.

118. Richter D, Klug B, Spielman A, Matuschka FR. Adaptation of diverse Lyme disease spirochetes in a natural rodent reservoir host. Infect Immun. 2004 Apr; 72 (4): 2442-4.

119. Skuballa J, Oehme R, Hartelt K, Petney T, Bucher T, Kimmig P, et al. European hedgehogs as hosts for *Borrelia* spp., Germany. Emerg Infect Dis. 2007 Jun; 13 (6): 952-3.

120. Kjelland V, Ytrehus B, Vikorren T, Stuen S, Skarpaas T, Slettan A. *Borrelia burgdorferi* sensu lato detected in skin of Norwegian mountain hares (*Lepus timidus*) without signs of dissemination. J Wildl Dis. 2011 Apr; 47 (2): 293-9.

121. Hanincova K, Taragelova V, Koci J, Schafer SM, Hails R, Ullmann AJ, et al. Association of *Borrelia garinii* and *B. valaisiana* with songbirds in Slovakia. Appl Environ Microbiol. 2003 May; 69 (5): 2825-30.

122. Comstedt P, Asokliene L, Eliasson I, Olsen B, Wallensten A, Bunikis J, et al. Complex population structure of Lyme borreliosis group spirochete *Borrelia garinii* in subarctic Eurasia. PLoS One. 2009; 4 (6): e5841.

123. Duneau D, Boulinier T, Gomez-Diaz E, Petersen A, Tveraa T, Barrett RT, et al. Prevalence and diversity of Lyme borreliosis bacteria in marine birds. Infect Genet Evol. 2008 May; 8 (3): 352-9.

124. Gylfe, Olsen B, Strasevicius D, Marti Ras N, Weihe P, Noppa L, et al. Isolation of Lyme disease *Borrelia* from puffins (*Fratercula arctica*) and seabird ticks (*Ixodes uriae*) on the Faeroe Islands. J Clin Microbiol. 1999 Apr; 37 (4): 890-6.

125. Amore G, Tomassone L, Grego E, Ragagli C, Bertolotti L, Nebbia P, et al. *Borrelia lusitaniae* in immature *Ixodes ricinus* (Acari: Ixodidae) feeding on common wall lizards in Tuscany, Central Italy. J Med Entomol. 2007 Mar; 44 (2): 303-7.

126. Richter D, Matuschka FR. Perpetuation of the Lyme disease spirochete *Borrelia lusitaniae* by lizards. Appl Environ Microbiol. 2006 Jul; 72 (7): 4627-32.

127. Heyman P, Cochez C, Hofhuis A, van der Giessen J, Sprong H, Porter SR, et al. A clear and present danger: tick-borne diseases in Europe. Expert Rev Anti Infect Ther. 2010 Jan; 8 (1): 33-50.

128. Health Board. Estonian communicable disease bulletins. http://www.terviseamet.ee/nakkushaigused/nakkushaigustesse-haigestumine.html, 03. August 2013.

129. Makinen J, Vuorinen I, Oksi J, Peltomaa M, He Q, Marjamaki M, et al. Prevalence of granulocytic *Ehrlichia* and *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks collected from Southwestern Finland and from Vormsi Island in Estonia. APMIS. 2003 Feb; 111 (2): 355-62.

130. Postic D, Korenberg E, Gorelova N, Kovalevski YV, Bellenger E, Baranton G. *Borrelia burgdorferi* sensu lato in Russia and neighbouring countries: high incidence of mixed isolates. Res Microbiol. 1997 Nov; 148 (8): 691-702.

131. Rauter C, Hartung T. Prevalence of *Borrelia burgdorferi* sensu lato genospecies in *Ixodes ricinus* ticks in Europe: a metaanalysis. Appl Environ Microbiol. 2005 Nov; 71 (11): 7203-16.

132. Wormser GP, Brisson D, Liveris D, Hanincova K, Sandigursky S, Nowakowski J, et al. *Borrelia burgdorferi* genotype predicts the capacity for hematogenous dissemination during early Lyme disease. J Infect Dis. 2008 Nov 1; 198 (9): 1358-64.

133. Steere AC. Lyme borreliosis in 2005, 30 years after initial observations in Lyme Connecticut. Wien Klin Wochenschr. 2006 Nov; 118 (21-22): 625-33.

134. Weber K. Aspects of Lyme borreliosis in Europe. Eur J Clin Microbiol Infect Dis. 2001 Jan; 20 (1): 6-13.

135. Wormser GP, Dattwyler RJ, Shapiro ED, Halperin JJ, Steere AC, Klempner MS, et al. 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. 2006 Nov 1; 43 (9): 1089-134.

136. Evans J. Lyme disease. Curr Opin Rheumatol. 2000 Jul; 12 (4): 311-7.

137. Bennet L, Fraenkel CJ, Garpmo U, Halling A, Ingman M, Ornstein K, et al. Clinical appearance of erythema migrans caused by *Borrelia afzelii* and *Borrelia garinii* – effect of the patient's sex. Wien Klin Wochenschr. 2006 Sep; 118 (17-18): 531-7.

138. Strle F, Nelson JA, Ruzic-Sabljic E, Cimperman J, Maraspin V, Lotric-Furlan S, et al. European Lyme borreliosis: 231 culture-confirmed cases involving patients with erythema migrans. Clin Infect Dis. 1996 Jul; 23 (1): 61-5.

139. Stanek G, Strle F. Lyme borreliosis. Lancet. 2003 Nov 15; 362 (9396): 1639-47.

140. Maraspin V, Cimperman J, Lotric-Furlan S, Ruzic-Sabljic E, Jurca T, Picken RN, et al. Solitary borrelial lymphocytoma in adult patients. Wien Klin Wochenschr. 2002 Jul 31; 114 (13-14): 515-23.

141. Ohlenbusch A, Matuschka FR, Richter D, Christen HJ, Thomssen R, Spielman A, et al. Etiology of the acrodermatitis chronica atrophicans lesion in Lyme disease. J Infect Dis. 1996 Aug; 174 (2): 421-3.

142. Asbrink E, Hovmark A. Lyme borreliosis: aspects of tick-borne *Borrelia burgdorferi* infection from a dermatologic viewpoint. Semin Dermatol. 1990 Dec; 9 (4): 277-91.

143. Mygland A, Ljostad U, Fingerle V, Rupprecht T, Schmutzhard E, Steiner I. EFNS guidelines on the diagnosis and management of European Lyme neuroborreliosis. Eur J Neurol. 2010 Jan; 17 (1): 8-16, e1-4.

144. Rudenko N, Golovchenko M, Grubhoffer L, Oliver JH, Jr. Updates on *Borrelia burgdorferi* sensu lato complex with respect to public health. Ticks Tick Borne Dis. 2011 Sep; 2 (3): 123-8.

145. Pachner AR, Steere AC. Neurological findings of Lyme disease. Yale J Biol Med. 1984 Jul-Aug; 57 (4): 481-3.

146. Stanek G, Fingerle V, Hunfeld KP, Jaulhac B, Kaiser R, Krause A, et al. Lyme borreliosis: clinical case definitions for diagnosis and management in Europe. Clin Microbiol Infect. 2011 Jan; 17 (1): 69-79.

147. Stanek G, Strle F. Lyme borreliosis: a European perspective on diagnosis and clinical management. Curr Opin Infect Dis. 2009 Oct; 22 (5): 450-4.

148. Mikkila HO, Seppala IJ, Viljanen MK, Peltomaa MP, Karma A. The expanding clinical spectrum of ocular Lyme borreliosis. Ophthalmology. 2000 Mar; 107 (3): 581-7.

149. Todorovic L, Ibisevic M, Alajbegovic A, Suljic-Mehmedika E, Jurisic V. Bilateral retrobulbar optic neuritis as first signs of Lyme disease. Med Arh. 2008; 62 (2): 117-8.

150. Chowdri HR, Gugliotta JL, Berardi VP, Goethert HK, Molloy PJ, Sterling SL, et al. *Borrelia miyamotoi* infection presenting as human granulocytic anaplasmosis: a case report. Ann Intern Med. 2013 Jul 2; 159 (1): 21-7.

151. Aguero-Rosenfeld ME, Wang G, Schwartz I, Wormser GP. Diagnosis of Lyme borreliosis. Clin Microbiol Rev. 2005 Jul; 18 (3): 484-509.

152. Tjernberg I, Henningsson AJ, Eliasson I, Forsberg P, Ernerudh J. Diagnostic performance of cerebrospinal fluid chemokine CXCL13 and antibodies to the C6-peptide in Lyme neuroborreliosis. J Infect. 2011 Feb; 62 (2): 149-58.

153. Lencakova D, Fingerle V, Stefancikova A, Schulte-Spechtel U, Petko B, Schreter I, et al. Evaluation of recombinant line immunoblot for detection of Lyme disease in Slovakia: comparison with two other immunoassays. Vector Borne Zoonotic Dis. 2008 Jun; 8 (3): 381-90.

154. Rizzoli A, Hauffe H, Carpi G, Vourc HG, Neteler M, Rosa R. Lyme borreliosis in Europe. Euro Surveill. 2011; 16 (27).

155. EUCALB. Treatment of Lyme borreliosis in Europe. http://meduni09.edis.at/eucalb/cms_15/index.php?option=com_content&view=article&id=44&Itemid=76, 30. April 2013

156. Postic D, Assous MV, Grimont PA, Baranton G. Diversity of *Borrelia burgdorferi* sensu lato evidenced by restriction fragment length polymorphism of rrf (5S)-rrl (23S) intergenic spacer amplicons. Int J Syst Bacteriol. 1994 Oct; 44 (4): 743-52.

157. Rar VA, Fomenko NV, Dobrotvorsky AK, Livanova NN, Rudakova SA, Fedorov EG, et al. Tickborne pathogen detection, Western Siberia, Russia. Emerg Infect Dis. 2005 Nov; 11 (11): 1708-15.

158. Schwaiger M, Cassinotti P. Development of a quantitative real-time RT-PCR assay with internal control for the laboratory detection of tick-borne encephalitis virus (TBEV) RNA. J Clin Virol. 2003 Jul; 27 (2): 136-45.

159. Skarpaas T, Golovljova I, Vene S, Ljostad U, Sjursen H, Plyusnin A, et al. Tickborne encephalitis virus, Norway and Denmark. Emerg Infect Dis. 2006 Jul; 12 (7): 1136-8.

160. Courtney JW, Kostelnik LM, Zeidner NS, Massung RF. Multiplex real-time PCR for detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi*. J Clin Microbiol. 2004 Jul; 42 (7): 3164-8.

161. Caporale DA, Rich SM, Spielman A, Telford SR, 3rd, Kocher TD. Discriminating between *Ixodes* ticks by means of mitochondrial DNA sequences. Mol Phylogenet Evol. 1995 Dec; 4 (4): 361-5.

162. Hall T. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series. 1999; 41: 95-8.

163. Tamura K. DJ, Nei M., and Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution. 2007; 24: 1596-9.

164. Newcombe RG. Two-sided confidence intervals for the single proportion: comparison of seven methods. Stat Med. 1998 Apr 30; 17 (8): 857-72.

165. Wilson EB. Probable Inference, the Law of Succession, and Statistical Inference. Journal of the American Statistical Association. 1927; 22: 209-12.

166. Fisher RA. On the Interpretation of $\chi 2$ from Contingency Tables, and the Calculation of P. Journal of the Royal Statistical Society. 1922 (85): 87-94.

167. Georgopoulou I, Tsiouris V. The potential role of migratory birds in the transmission of zoonoses. Vet Ital. 2008 Oct-Dec; 44 (4): 671-7.

168. Waldenstrom J, Lundkvist A, Falk KI, Garpmo U, Bergstrom S, Lindegren G, et al. Migrating birds and tickborne encephalitis virus. Emerg Infect Dis. 2007 Aug; 13 (8): 1215-8.

169. Jaaskelainen AE, Sironen T, Murueva GB, Subbotina N, Alekseev AN, Castren J, et al. Tick-borne encephalitis virus in ticks in Finland, Russian Karelia and Buryatia. J Gen Virol. 2010 Nov; 91 (Pt 11): 2706-12.

170. Comstedt P, Bergstrom S, Olsen B, Garpmo U, Marjavaara L, Mejlon H, et al. Migratory passerine birds as reservoirs of Lyme borreliosis in Europe. Emerg Infect Dis. 2006 Jul; 12 (7): 1087-95.

171. Hasle G, Bjune GA, Midthjell L, Roed KH, Leinaas HP. Transport of *Ixodes ricinus* infected with *Borrelia* species to Norway by northward-migrating passerine birds. Ticks Tick Borne Dis. 2011 Mar; 2 (1): 37-43.

172. Dubska L, Literak I, Kocianova E, Taragelova V, Sychra O. Differential role of passerine birds in distribution of *Borrelia* spirochetes, based on data from ticks collected from birds during the postbreeding migration period in Central Europe. Appl Environ Microbiol. 2009 Feb; 75 (3): 596-602.

173. Kipp S, Goedecke A, Dorn W, Wilske B, Fingerle V. Role of birds in Thuringia, Germany, in the natural cycle of *Borrelia burgdorferi* sensu lato, the Lyme disease spirochaete. Int J Med Microbiol. 2006 May; 296 Suppl 40: 125-8.

174. Poupon M, Lommano E, Humair PF, Douet V, Rais O, Schaad M, et al. Prevalence of *Borrelia burgdorferi* sensu lato in ticks collected from migratory birds in Switzerland. Appl Environ Microbiol. 2006 Jan; 72 (1): 976-9.

175. Derdakova M, Beati L, Pet'ko B, Stanko M, Fish D. Genetic variability within *Borrelia burgdorferi* sensu lato genospecies established by PCR-single-strand conformation polymorphism analysis of the rrfA-rrlB intergenic spacer in *Ixodes ricinus* ticks from the Czech Republic. Appl Environ Microbiol. 2003 Jan; 69 (1): 509-16.

176. Gern L, Douet V, Lopez Z, Rais O, Moran Cadenas F. Diversity of *Borrelia* genospecies in *Ixodes ricinus* ticks in a Lyme borreliosis endemic area in Switzerland identified by using new probes for reverse line blotting. Ticks Tick Borne Dis. 2010 Mar; 1 (1): 23-9.

177. Kurtenbach K, Schafer SM, Sewell HS, Peacey M, Hoodless A, Nuttall PA, et al. Differential survival of Lyme borreliosis spirochetes in ticks that feed on birds. Infect Immun. 2002 Oct; 70 (10): 5893-5.

178. Franke J, Meier F, Moldenhauer A, Straube E, Dorn W, Hildebrandt A. Established and emerging pathogens in *Ixodes ricinus* ticks collected from birds on a conservation island in the Baltic Sea. Med Vet Entomol. 2010 Dec; 24 (4): 425-32.

179. Humair PF, Postic D, Wallich R, Gern L. An avian reservoir (*Turdus merula*) of the Lyme borreliosis spirochetes. Zentralbl Bakteriol. 1998 May; 287 (4): 521-38.

180. Katargina O, Geller J, Alekseev A, Dubinina H, Efremova G, Mishaeva N, et al. Identification of *Anaplasma phagocytophilum* in tick populations in Estonia, the European part of Russia and Belarus. Clin Microbiol Infect. 2012 Jan; 18 (1): 40-6.

181. Borgoiakov V, Fomenko NV, Panov VV, Chikova ED. [Study on the infection of taiga ticks with *Borrelia* in the territory of Novosibirsk Scientific Center SB PAS]. Parazitologiia. 2011 Nov-Dec; 44 (6): 543-56.

182. Richter D, Schlee DB, Matuschka FR. Relapsing fever-like spirochetes infecting European vector tick of Lyme disease agent. Emerg Infect Dis. 2003 Jun; 9 (6): 697-701.

183. Davis S, Bent SJ. Loop analysis for pathogens: niche partitioning in the transmission graph for pathogens of the North American tick *Ixodes scapularis*. J Theor Biol. 2011 Jan 21; 269 (1): 96-103.

184. Motiejunas L, Bunikis J, Barbour AG, Sadziene A. Lyme borreliosis in Lithuania. Scand J Infect Dis. 1994; 26 (2): 149-55.

185. Reye AL, Stegniy V, Mishaeva NP, Velhin S, Hubschen JM, Ignatyev G, et al. Prevalence of tick-borne pathogens in *Ixodes ricinus* and *Dermacentor reticulatus* ticks from different geographical locations in Belarus. PLoS One. 2013; 8 (1): e54476.

186. Estrada-Pena A, Farkas R, Jaenson TG, Koenen F, Madder M, Pascucci I, et al. Association of environmental traits with the geographic ranges of ticks (Acari: Ixodidae) of medical and veterinary importance in the western Palearctic. A digital data set. Exp Appl Acarol. 2013 Mar; 59 (3): 351-66.

187. Etti S, Hails R, Schafer SM, De Michelis S, Sewell HS, Bormane A, et al. Habitat-specific diversity of *Borrelia burgdorferi* sensu lato in Europe, exemplified by data from Latvia. Appl Environ Microbiol. 2003 May; 69 (5): 3008-10.

188. Junttila J, Peltomaa M, Soini H, Marjamaki M, Viljanen MK. Prevalence of *Borrelia burgdorferi* in *Ixodes ricinus* ticks in urban recreational areas of Helsinki. J Clin Microbiol. 1999 May; 37 (5): 1361-5.

189. Korotkov I, S., Kislenko GS, Burenkova LA, Rudnikova NA, Karan LS. [Spatial and temporal variability of *Ixodes ricinus* and *Ixodes persulcatus* infection with the Lyme disease agent in Moscow Region]. Parazitologiia. 2008 Nov-Dec; 42 (6): 441-51.

190. Alekseev AN, Dubinina HV, Van De Pol I, Schouls LM. Identification of *Ehrlichia* spp. and *Borrelia burgdorferi* in *Ixodes* ticks in the Baltic regions of Russia. J Clin Microbiol. 2001 Jun; 39 (6): 2237-42.

191. Bormane A, Lucenko I, Duks A, Mavtchoutko V, Ranka R, Salmina K, et al. Vectors of tick-borne diseases and epidemiological situation in Latvia in 1993-2002. Int J Med Microbiol. 2004 Apr; 293 Suppl 37: 36-47.

192. Franke J, Hildebrandt A, Dorn W. Exploring gaps in our knowledge on Lyme borreliosis spirochaetes--updates on complex heterogeneity, ecology, and pathogenicity. Ticks Tick Borne Dis. 2013 Feb; 4 (1-2): 11-25.

193. Lommano E, Bertaiola, L., Dupasquier, C., Gern, L. Infections and co-infections of questing *Ixodes ricinus* ticks by emerging zoonotic pathogens in Western Switzerland. Appl Environ Microbiol. 2012 Jul; 78 (13): 4606-12.

194. Skuballa J, Petney T, Pfaffle M, Oehme R, Hartelt K, Fingerle V, et al. Occurrence of different *Borrelia burgdorferi* sensu lato genospecies including *B. afzelii, B. bavariensis*, and *B. spielmanii* in hedgehogs (*Erinaceus* spp.) in Europe. Ticks Tick Borne Dis. 2012 Feb; 3 (1): 8-13.

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SUMMARY

The most common tick-borne disease in the temperate zone is Lyme borreliosis (LB) or tick-borne borreliosis: tens of thousands cases are registered in both in the USA and Europe every year. In Estonia, the morbidity related to LB has increased drastically over the last decade, reaching its maximum value of 171.8 cases per 100 000 population in 2011. This number is one of the highest in Europe and the highest in Baltic countries. The causative agents for this disease are spirochetes of *Borrelia burgdorferi* s.l. complex that are maintaining in nature in various animal hosts, primarily rodents and birds, and can be transmitted to humans via the bite of a tick, which had got infection after feeding on an infected animal host. It has been recently reported that *B. miyamotoi,* belonging to the relapsing fever group, also causes infections in humans. Estonia is situated in the unique area of overlapping ranges of two main tick vector species in Europe, *Ixodes ricinus* and *I. persulcatus*, providing thus a special opportunity to study and investigate circulation, prevalence and genetic characteristics of TBPs. The northern and western regions of Estonia are allopatric only for *I. ricinus* distribution, while the southern and eastern regions are sympatric for both tick species.

The investigation of *I. ricinus* ticks removed from migratory passerines during postbreeding migration period revealed the presence of medically important European subtype of TBEV, *B. garinii*, *B. valaisiana*, *B. afzelii* and *Anaplasma phagocytophilum*. All TBPs, except TBEV, were detected in ticks removed from short migrants of *Turdus* sp., common blackbirds and redwings. The results of the study indicate the potentially important role migratory birds may play in the dissemination of ticks and TBPs over the long distances between hemispheres and continents. Birds may serve as an infection source, as tick transporters and also infection transmitters between ticks by co-feeding.

The investigation of *Borrelia* genospecies in Estonian tick populations showed for the first time in Estonia the circulation of Lyme borreliosis group spirochetes of medical importance *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. bavariensis* and *B. burgdorferi* s.s. as well as relapsing fever group spirochete *B. miyamotoi*, which is also known to cause a disease in humans. For *B. miyamotoi* the prevalence varied from 0.6% to 2.8% and for *B. burgdorferi* s.l. from 4.9% to 17.5%. Significant differences were demonstrated in infection rates between *I. ricinus* and *I. persulcatus* ticks as well as between areas of *I. ricinus* allopatry and sympatry for both tick species. Phylogenetic analysis revealed that Estonian sequences cluster with sequences detected in ticks, animals and humans in European (incl. Russia, Ukraine and Belarus) as well as Asian countries. The detection of *B. valaisiana* in unspecific vector *I. persulcatus* and Asian type of *B. miyamotoi* in *I. ricinus* within sympatric area may represent the exchange of pathogens between vectors which

may lead to the adaptation to new vector species and introduction of the pathogen into new foci. The areas with highest *Borrelia* prevalence in ticks in Estonia are Ida-Virumaaa, Valgamaa, Tartumaa and Saaremaa.

KOKKUVÕTE

Puukborrelioos ehk Lyme'i tõbi on kõige levinum puukidega ülekantav haigus parasvöötmes. Nii Euroopas kui ka USAs registreeritakse igal aastal kümneid tuhandeid haigusjuhtumeid. Puukborrelioosi haigestumine on Eestis viimase aastakümne jooksul märkimisväärselt tõusnud, saavutades kõrgpunkti 2011. aastal, mil registreeriti 171,8 haigusjuhtumit 100 000 elaniku kohta. See näitaja on üks kõrgematest Euroopas ja kõrgeim Baltimaades. Puukborrelioosi põhjustajateks on *Borrelia burgdorferi* s.l. kompleksi kuuluvad spiroheedid, mille looduslikeks reservuaarideks on loomad, peamiselt närilised, ning linnud. Inimesele võib puukborrelioos üle kanduda patogeeni kandvatel loomadel toitunud puugi hammustusega. Hiljuti selgus, et taastuvat palavikku tekitav *Borrelia* bakter *B. miyamotoi* on ka inimesele patogeenne. Nii *B. burgdorferi* s.l. kui ka *B. miyamotoi* kasutavad ülekandevektoritena puuke perekonnast *Ixodes*. Eesti on unikaalne piirkond Euroopas, kus levib kaks peamist puukülekandjat, *Ixodes ricinus* ja *I. persulcatus*. Selline asukoht annab erakordse võimaluse uurida puukidega ülekantavate haigustekitajate ringlust, levimust ja geneetilisi iseärasusi. *Ixodes ricinus* ehk võsapuuk on laialt levinud Euroopas ning teda leidub ka kõikjal Eestis, *I. persulcatus* ehk laanepuuk on levinud Põhja-Venemaal ja Aasias, Eestis on levialaks maa lõuna- ja idaosa.

Sügismigratsiooni perioodil leiti värvuliste seltsi kuuluvatelt rändlindudelt eemaldatud võsapuukides *I. ricinus* inimesele patogeenseid haigustekitajaid *B. garinii*, *B. afzelii* ja *B. valaisiana*, mis põhjustatavad puukentsefaliiti ja puukborrelioosi, ning anaplasmoosi tekitavat *Anaplasma phagocytophilum*'it. Suurem osa nimetatud haigustekitajatest leiti puukidelt, kes olid eemaldatud rästaslaste perekonna lähiränduritelt, must- ja vainurästastelt. Uuringu tulemused viitavad olulisele rollile, mida rändlinnud võivad etendada puukide ja puukidega ülekantavate haigustekitajate levitamisel lindude rändesuunas mandrite ja poolkerade vahel. Linnud võivad olla nii nakkusallikaks kui ka puukide transportijaiks, aga ka soodustada patogeenide ülekannet puukide vahel kaastoitumise teel.

Borrelia perekonda kuuluvate bakterite uurimine näitas esmakordselt Eestis, et kohalikus puugipopulatsioonis on levinud nii Lyme'i tõve põhjustavad, meditsiiniliselt oluliste spiroheetide hulka kuuluvad *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. bavariensis* ja *B. burgdorferi* s.s. kui ka inimesel taastuvat palavikku esilekutsuv *B. miyamotoi*. Kogu populatsioonis osutus *Borrelia burgdorferi* s.l. levimuseks 4,9% kuni 17,5%, *B. miyamotoi* levimuseks 0,6% kuni 2,8%. Märkimisväärsed erinevused puukide infitseeritavuses tuvastati nii kahe puugiliigi vahel kui ka regioonide vahel, kus elutseb üksnes *I. ricinus* või mõlemad puugiliigid. Fülogeneetilise analüüsi käigus selgus, et *Borrelia* Eesti tüvede järjestused on kõrgel määral sarnased Euroopa (sh Venemaa, Ukraina ja Valgevene) ning Aasia riikidest pärit puukides, loomades ja inimestes

leitutega. *Borrelia valaisiana* tuvastamine puukides *I. persulcatus* ning ka *B. miyamotoi* Aasia-tüübi avastamine puukides *I. ricinus* aladel, kus leidub mõlemat puugiliiki, võib viidata patogeenide vahetumisele ülekandjate vahel, kohandumisele uue ülekandjaliigiga ning haigustekitaja levimisele uutele aladele. Antud töö käigus selgusid piirkonnad, kus on kõrge risk sattuda *Borrelia*-nakatatud puugile, need on Ida-Virumaa, Valga-, Tartu- ja Saaremaa.

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Education

2006 –	Tallinn University of Technology, PhD student
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Special courses

June 2010	Basic Immunology Course, directed by prof. Abul K. Abbas, University of California, San Francisco. Tallinn, Estonia.
November, 2009	Infectious disease diagnostics seminar (Siemens) (5 educational points), Tallinn, 2009

Professional employment

2012 –	National Institute for Health Development, Virology Department, researcher
2006 – 2012	National Institute for Health Development, Virology Department, researcher's assistant
July – December 2005	HIV Reference laboratory, trainee

Research activity, awards and honors

2013	Oral presentation at XII International Jena Symposium on Tick-borne Diseases, Weimar, 21–23 March, 2009 – Sinnecker-Kunz Award for Young Scientists, 3rd Prize.
2012	Oral presentation at Estonian Teriology Society Autumn School, 12 October 2012
2011	Oral presentation at workshop Disease vectors today: Changes in ecology, climate and public health risks, 24–27

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2010	Oral presentation, final conference of EDEN project, Montpellier, France, 23–25 May, 2010 – DoRa T8 award Poster presentation at 12th International Conference on Lyme Borreliosis and other Tick-Borne Diseases, 26–29 September, 2010 – DoRa T8 award
2009	X International Jena Symposium on Tick-borne Diseases , Weimar, 19–21 March, 2009 Annual meeting of VISBY Sweden-Russia-Belarus network "Anthropogenic pressure, changing tick population and increasing risk of tick-borne emerging diseases", Tallinn, April
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Publications

Geller J, Nazarova L, Katargina O, Golovljova I. *Borrelia burgdorferi* sensu lato prevalence in tick populations in Estonia. Parasit Vectors. 2013 Jul 9; 6: 202.

Katargina O, Russakova S, **Geller J**, Kondrusik M, Zajkowska J, Zygutiene M, Bormane A, Trofimova J, Golovljova I. Detection and characterization of tick-borne encephalitis virus in Baltic countries and Eastern Poland. PLoS One. 2013 May 1; 8(5)

Geller J, Nazarova L, Katargina O, Leivits A, Järvekülg L, Golovljova I. Tick-borne pathogens in ticks feeding on migratory passerines in Western part of Estonia. Vector Borne Zoonotic Dis. 2013 Jul; 13 (7): 443-8.

Geller J, Nazarova L, Katargina O, Järvekülg L, Fomenko N, Golovljova I. Detection and genetic characterization of relapsing fever spirochete *Borrelia miyamotoi* in Estonian ticks. PLoS One. 2012; 7 (12): e51914.

Katargina O, **Geller J**, Alekseev A, Dubinina H, Efremova G, Mishaeva N, Vasilenko V, Kuznetsova T, Järvekülg L, Vene S, Lundkvist A, Golovljova I. Identification of *Anaplasma phagocytophilum* in tick populations in Estonia, the European part of Russia and Belarus. Clin Microbiol Infect. 2012 Jan; 18 (1): 40-6.

Katargina O, **Geller J**, Vasilenko V, Kuznetsova T, Järvekülg L, Vene S, Lundkvist Å, Golovljova I. Detection and characterization of *Babesia* species in *Ixodes* ticks in Estonia. Vector Borne Zoonotic Dis. 2011 Jul; 11 (7): 923-8.

Golovljova I, Katargina O, **Geller J**, Tallo T, Mittzenkov V, Vene S, Nemirov K, Kutsenko A, Kilosanidze G, Vasilenko V, Plyusnin A, Lundkvist A. Unique signature amino acid substitution in Baltic tick-borne encephalitis virus (TBEV) strains within the Siberian TBEV sybtype. International Journal of Medical Microbiology. 2008; 298S1(44), 108 - 120.

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Geller J, Nazarova L, Katargina O, Golovljova I. *Borrelia burgdorferi* sensu lato prevalence in tick populations in Estonia. Parasit Vectors. 2013 Jul 9; 6: 202.

Katargina O, Russakova S, **Geller J**, Kondrusik M, Zajkowska J, Zygutiene M, Bormane A, Trofimova J, Golovljova I. Detection and characterization of tick-borne encephalitis virus in Baltic countries and Eastern Poland. PLoS One. 2013 May 1; 8(5)

Geller J, Nazarova L, Katargina O, Leivits A, Järvekülg L, Golovljova I. Tick-borne pathogens in ticks feeding on migratory passerines in Western part of Estonia. Vector Borne Zoonotic Dis. 2013 Jul; 13 (7): 443-8.

Geller J, Nazarova L, Katargina O, Järvekülg L, Fomenko N, Golovljova I. Detection and genetic characterization of relapsing fever spirochete *Borrelia miyamotoi* in Estonian ticks. PLoS One. 2012; 7 (12): e51914.

Katargina O, **Geller J**, Alekseev A, Dubinina H, Efremova G, Mishaeva N, Vasilenko V, Kuznetsova T, Järvekülg L, Vene S, Lundkvist A, Golovljova I. Identification of *Anaplasma phagocytophilum* in tick populations in Estonia, the European part of Russia and Belarus. Clin Microbiol Infect. 2012 Jan; 18 (1): 40-6.

Katargina O, **Geller J**, Vasilenko V, Kuznetsova T, Järvekülg L, Vene S, Lundkvist Å, Golovljova I. Detection and characterization of *Babesia* species in *Ixodes* ticks in Estonia. Vector Borne Zoonotic Dis. 2011 Jul; 11 (7): 923-8.

Golovljova I, Katargina O, **Geller J**, Tallo T, Mittzenkov V, Vene S, Nemirov K, Kutsenko A, Kilosanidze G, Vasilenko V, Plyusnin A, Lundkvist A. Unique signature amino acid substitution in Baltic tick-borne encephalitis virus (TBEV) strains within the Siberian TBEV sybtype. International Journal of Medical Microbiology. 2008; 298S1(44), 108 - 120.

PUBLICATION I

Tick-borne pathogens in ticks feeding on migratory passerines in Western part of Estonia

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ORIGINAL ARTICLE

Tick-Borne Pathogens in Ticks Feeding on Migratory Passerines in Western Part of Estonia

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Abstract

During southward migration in the years 2006–2009, 178 migratory passerines of 24 bird species infested with ticks were captured at bird stations in Western Estonia. In total, 249 nymphal ticks were removed and analyzed individually for the presence of *Borrelia burgdorferi* sensu lato (s.l.), tick-borne encephalitis virus (TBEV), and *Anaplasma phagocytophilum*. The majority of ticks were collected from *Acrocephalus* (58%), *Turdus* (13%), *Sylvia* (8%), and *Parus* (6%) bird species. Tick-borne pathogens were detected in nymphs removed from *Acrocephalus*, *Turdus*, and *Parus* bird species. TBEV of the European subtype was detected in 1 *I. ricinus* nymph removed from *A. palustris*. *B. burgdorferi* s.l. DNA was found in 11 ticks (4.4%) collected from *Turdus* and *Parus* species. Bird-associated *B. garinii* and *B. valaisiana* were detected in *I. ricinus* nymphs removed from *T. merula*. Rodent-associated *B. afzelii* was detected in 3 *I. ricinus* nymphs from 2 *P. major* birds. One of the *B. afzelii*-positive nymphs was infected with a mix of 2 *B. afzelii* strains, whereas 1 of these strains was also detected in another nymph feeding on the same great tit. The sharing of the same *B. afzelii* strain by 2 nymphs indicates a possible transmission of *B. afzelii* by co-feeding on a bird. *A. phagocytophilum* DNA was detected in 1 *I. ricinus* nymph feeding on a *T. iliacus*. The results of the study confirm the possible role of migratory birds in the dispersal of ticks infected with tick-borne pathogens along the southward migration route via Estonia.

Key Words: Tick-borne pathogen—TBEV—Borrelia—Reservoir—tick—Ixodidae—Bird.

Introduction

Migratory birds may play an important role in global dispersal, transport, and dissemination of ticks and tick-borne pathogens as they migrate within and between continents (Georgopoulou and Tsiouris 2008). Birds can carry ticks infected by such tick-borne pathogens as tick-borne encephalitis virus (TBEV) (Waldenstrom et al. 2007), *Anaplasma phagocytophilum* (Alekseev et al. 2001, Bjoersdorff et al. 2001, Daniels et al. 2002, Franke et al. 2010a) and *Borrelia burgdorferi* sensu lato (s.l.) (Olsen et al. 1995, Gylfe et al. 2000, Hanincova et al. 2003b, Poupon et al. 2006, Franke et al. 2010a).

Ixodes ricinus and *I. persulcatus* are the most important vectors for tick-borne pathogens in Europe and Asia, respectively. Estonia is located in a unique area where both tick species co-circulate. Our previous studies showed that all three known TBEV subtypes (Suss 2011), Western TBEV (W-TBEV), Far-Eastern TBEV (FE-TBEV), and Siberian TBEV (S-TBEV), co-circulate in Estonia (Golovljova et al. 2004). The prevalence of *B. burgdorferi* s.l. in the tick population varied from 3.8% to 23% (mean 9.7%) in different regions of Estonia (Geller, unpublished). *A. phagocytophilum* was found in questing *I. ricinus* ticks with a prevalence of 1.7% for mainland Estonia and 2.6% for Saaremaa island (Katargina et al. 2012).

Estonia is situated in the breeding area of long-distance migrating passerines, such as *Acrocephalus* spp., that might be potentially important in the dispersal of ticks and tick-borne pathogens along its southward migration route from Estonia, Fennoscandia, and southeastern Russia to Africa (A. Leivits, personal communication). Short-distance migrants of *Turdus* species, which are known to be reservoirs (Hanincova et al. 2003b, Humair et al. 1998) for pathogenic *B. garinii* and potentially pathogenic *B. valaisiana,* also breed in Estonia, Finland, and southeastern Russia. In this study, we investigated the prevalence of TBEV, *B. burgdorferi* s.l., and *A. phagocytophilum* in ticks harbored by passerines during southward migration. This is the first study regarding the role of birds in the dispersal of these pathogens in Estonia.

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Materials and Methods

Collection of ticks from migratory birds

Birds were captured by ornithologists in Kabli (58.01431N 24.44942 E) and Pulgoja (58.09947N 24.4844E) bird stations situated on the East coast of the Gulf of Riga. Birds were trapped during the fall migration periods in August–October from 2006 to 2009. The birds were identified, examined, and released back into the wild as quickly as possible to minimize stress. Only birds from which ticks had been removed were included in the study. Ticks were collected from the bird's head using tweezers and placed in 70% ethanol. Samples were sent to the laboratory within 1 month after ticks' collection and processed immediately. The tick species were identified morphologically by use of a stereomicroscope. Ticks were washed in sterile water and homogenized in 300 μL phosphate-buffered saline (PBS) solution with TissueLyzer (Retsch, Haan, Germany). Two hundred microliters of suspensions were used for nucleic acid extraction. Due to small numbers, larvae collected from birds were not included in the study.

Nucleic acid isolation

Nucleic acids were extracted by the guanidinium thiocyanate–phenol–chloroform method using the TriPure isolation system (Roche Diagnostics, Lewes, UK) according to the manufacturer's recommendations. Sterile water was used as a negative control for every preparation set. DNA was stored at -20°C and RNA at -70°C until further use.

To avoid any possible contamination, all steps were performed in separate rooms with sterile techniques, and addition of the sample DNA to the PCR mix was performed in a laminar flow cabinet. All primers and probes used in the current study are presented in Table 1. T1 ▶

Detection of tick-borne encephalitis virus RNA

TBEV real-time reverse transcriptase PCR detection in ticks was carried out with F-TBE1 and R-TBE1 primers and TBE-probe-WT probe as described (Schwaiger and Cassinotti 2003) with some modifications. Forward primer concentration of 3 μM, reverse primer concentration of 0.3 μM, and probe concentration of 0.2 μM were used, and the reverse transcription step was performed at 42°C. The 7900 Real Time PCR system (Applied Biosystems) was used for all PCR reactions and fluorescent detections.

Samples positive for TBEV RNA by real-time PCR were amplified and sequenced for the partial E gene, as described earlier (Skarpaas et al. 2006) with outer primers 283F1 and 827R1 used for the cDNA synthesis and inner primers 349F2 and 814R2 for the second round of PCR amplification.

Detection of B. burgdorferi *s.l. DNA*

All samples were screened for the presence of *B. burgdorferi* s.l. by amplification of the 5S–23S rRNA intergenic spacer (IGS) region as described earlier (Postic et al. 1994, Rar et al. 2005), with modified cycling conditions. In the first-round PCR, the annealing step at 58°C was increased to 1 min and the extension step at 72°C was increased to 2 min. In the nested PCR, cycling conditions were performed as described (Postic et al. 1994).

The amplified products were separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide and visualized under ultraviolet (UV) light.

Detection of A. phagocytophilum *DNA*

Ticks were screened for the presence of *A. phagocytophilum* by real-time PCR with ApMSP2f and ApMSP2r primers and ApMSP2 probe, as described previously (Courtney et al. 2004).

DNA sequencing and data analysis

Sequencing was performed by the Estonian Biocentre (Tartu, Estonia), where PCR products were sent. Assembling, editing, and analysis of collected sequences were performed using BioEdit Sequences Alignment Editor v.7.0.9.0.

Table 1. Primers and Probes Used for PCR, RT-PCR, and Real-Time PCR of Tick-Borne Pathogen Nucleic Acid Detection

Primer or probe name	*Sequences of oligonucleotide probes and primers (5′→3′)*	*References*
TBEV		
283F1	GAGAYCAGAGTGAYCGAGGCTGG	Skarpaas et al. 2006
827R1	AGGTGGTACTTGGTTCCMTCAAGT	
349F2	GTCAAGGCGKCTTGTGAGGCAA	
814R2	TTCCCTCAATGTGTGCCACAGG	
F-TBE1	GGGCGGTTCTTGTTCTCC	Schwaiger and Cassinotti 2003
R-TBE1	ACACATCACCTCCTTGTCAGACT	
TBE-Probe-WT	TGAGCCACCATCACCCAGACACA	
B. burgdorferi s.l.		
NC1	CCTGTTATCATTCCGAACACAG	Rar et al. 2005
NC2	TACTCCATTCGGTAATCTTGGG	
NC3	CTGCGAGTTCGCGGGAGA	Postic et al. 1994
NC4	TCCTAGGCATTCACCATA	
A. phagocytophilum		
ApMSP2f	ATGGAAGGTAGTGTTGGTTATGGTATT	Courtney et al. 2004
ApMSP2r	TTGGTCTTGAAGCGCTCGTA	
ApMSP2 (probe)	FAM-TGGTGCCAGGGTTGAGCTTGAGATTG-TAMRA	

TBEV, tick-borne encephalitis virus.

Table 2. Birds Captured in Estonia During the Summer—Autumn Migration Period and Their Infestation with *I. ricinus* Ticks

Bird species		*No. of birds*	*% of all birds*	*No. of ticks collected*
Great reed-warbler	*Acrocephalus arundinaceus*	1	0.6	1
Blyth's reed-warbler	*A. dumetorum*	9	5.1	11
Marsh warbler	*A. palustris*	61	34.3	99
Sedge warbler	*A. schoenobaenus*	7	3.9	6[a]
Eurasian reed-warbler	*A. scirpaceus*	25	14.0	28
Tree pipit	*Anthus trivialis*	2	1.1	7
Common rosefinch	*Carpodacus erythrinus*	1	0.6	4
Eurasian treecreeper	*Certhia familiaris*	2	1.1	2
Reed bunting	*Emberiza schoeniclus*	1	0.6	1
European robin	*Erithacus rubecula*	2	1.1	1
Red-backed shrike	*Lanius collurio*	1	0.6	1
Common grasshopper-warbler	*Locustella naevia*	1	0.6	1
Blue tit	*Parus caeruleus*	2	1.1	2
Great tit	*P. major*	14	7.9	14
Common redstart	*Phoenicurus phoenicurus*	1	0.6	1
Common chiffchaff	*Phylloscopus collybita*	10	5.6	10
Willow warbler	*Ph. trochilus*	4	2.2	5
Goldcrest	*Regulus regulus*	1	0.6	1
Garden warbler	*Sylvia borin*	1	0.6	1
Whitethroat	*S. communis*	14	7.9	18
Winter wren	*Troglodytes troglodytes*	2	1.1	2
Redwing	*Turdus iliacus*	2	1.1	2
Song thrush	*T. philomeros*	2	1.1	2
Common blackbird	*T. merula*	12	6.7	29
Total		178		249

[a]One *I. persulcatus* nymph is included.

Results

Examination of birds and ticks

In total, 178 birds of 24 bird species infested with ticks were examined in the study. All birds were juvenile passerines (order *Passeriformes*). Only 1 bird (great tit) was identified by ornithologists as an adult (>1 year old). The most common birds were the marsh warbler (*A. palustris*), the Eurasian reed-warbler (*A. scirpaceus*), and the great tit (*P. major*), comprising 34.3%, 14%, and 7.9% of all birds, respectively. All details concerning the captured birds, their species and the number of ticks removed from them are presented in Table 2. T2 ▶

Overall, 249 nymphs were removed from the examined birds, of which 248 (99.6 %) were morphologically identified as *I. ricinus* and 1 (0.4%) as *I. persulcatus* (removed from *A. schoenobaenus*, sedge warbler). The majority of ticks (58%) were collected from *Acrocephalus*, *Turdus* (13%), *Sylvia* (8%), and *Parus* (6%) bird species.

Tick-borne pathogens detected in ticks

All ticks were investigated for the presence of TBEV, *B. burgdorferi* s.l., and *A. phagocytophilum*. Tick-borne pathogens were detected in nymphs removed from *Acrocephalus*, *Turdus*, and *Parus* bird species. Overall, 13 ticks (5.2%) removed from 7 birds were infected by tick-borne pathogens (Table 3). ◀ T3

TBEV detection

TBEV RNA was detected only in 1 tick (0.4% of all examined ticks) removed from a marsh warbler. Another 4 nymphs attached to the same bird were negative for TBEV. Phylogenetic analysis of partial E gene showed that it belonged to the TBEV-Eu subtype and is closely related to the strain Korppoo-259 from Finland (Jaaskelainen et al. 2010).

Detection of B. burgdorferi *s.l.*

B. burgdorferi s.l. DNA was detected in 11 ticks (4.4% of all examined ticks) removed from 5 birds of 2 species (common

Table 3. Tick-Borne Pathogens in Ticks Removed from Birds

Bird species	*Infested tick species*	*No. ticks infected/no. ticks on bird*	*Infectious agent*
Marsh warbler *(A. palustris)*	*I. ricinus*	1N/4N	TBEV-Eu
Great tit *(P. major)*	*I. ricinus*	2N/2N	*B. afzelii*
Great tit *(P. major)*	*I. ricinus*	1N/1N	*B. afzelii*
Common blackbird *(T. merula)*	*I. ricinus*	4N/5N	*B. garinii*
Common blackbird *(T. merula)*	*I. ricinus*	3N/3N	*B. garinii*
Common blackbird *(T. merula)*	*I. ricinus*	1N/1N	*B. valaisiana*
Redwing *(T. iliacus)*	*I. ricinus*	1N/1N	*A. phagocytophilum*

TBEV, tick-borne encephalitis virus.

blackbird and great tit). *B. garinii* was detected in 7 *I. ricinus* nymphs removed from 2 common blackbirds. One common blackbird carried 1 *B.valaisiana*–positive nymph. Analysis of the 5S–23S IGS showed that the sequences were identical to those of *B. garinii* 20047 (Postic et al. 1994) and NE11 (Gern et al. 2010), and to the sequence of *B. valaisiana* genotype I-214 (Derdakova et al. 2003). Three *I. ricinus* nymph samples from 2 birds of *P. major* (great tit) were identified as *B. afzelii*. Two *I. ricinus* nymphs recovered from the same great tit were infected by 2 different types of *B. afzelii*. One nymph had a mixture of 2 sequences (*B. afzelii* strain PGau and *B. afzelii* strain Tom1503), and the sequence obtained from another nymph was identical only to *B. afzelii* strain PGau.

Despite the large number of ticks (145/249, 58%) removed from *Acrocephalus* species none of them were infected with *B. burgdorferi* s.l.

Detection of A. phagocytophilum

Only 1 *I. ricinus* nymph (0.4% of all examined ticks), removed from a redwing (*T. iliacus*) was positive for *A. phagocytophilum* by real-time PCR analysis with a cycle threshold (Ct) value of 25.3.

Discussion

Thousands of passerines migrate southward every autumn from their places of breeding in Estonia, Finland, and northwestern Russia to their wintering areas located in Southeast Asia, Europe, and Africa. The present study confirms the potential role of migratory passerines in the distribution of tick-borne pathogens by harboring infected ticks, as we found a TBEV-infected nymph harbored by *Acrocephalus* species, and ticks infected by *Borrelia* and *A. phagocytophilum* that were removed from *Turdus* species.

Birds carrying TBEV infected ticks may contribute to the introduction of TBEV and thereby to the emergence of new TBE foci (Lommano et al. 2012). Data from Sweden showed 0.5% TBEV prevalence in bird-feeding ticks removed from the tree pipit (*Anthus trivialis*), the European robin (*Erithacus rubecula*), the common redstart (*Phoenicurus phoenicurus*), and the song thrush (*T. philomeros*) (Waldenstrom et al. 2007). The prevalence of TBEV in ticks from birds in the current study was the similar (0.4%). A TBEV-positive *I. ricinus* nymph was removed from a marsh warbler (*A. palustris*), indicating a potential implication of *Acrocephalus* species in the dispersal of TBEV-infected nymphs from breeding areas in Estonia, Finland, and northwestern Russia along the route to the wintering areas in Central and South Africa. Although there were 4 nymphs attached to the same bird, only 1 of them was TBEV positive, indicating that transmission by co-feeding did not occur on this host. The close sequence relationship to the TBEV strain reported from Finland (Jaaskelainen et al. 2010) could be explained by the marsh warbler migration route, which is directed southward from their breeding areas in South Finland through Estonia to South Africa.

In Europe the prevalence of *B. burgdorferi* s.l spirochetes in *I. ricinus* nymphs feeding on bird ticks varies considerably. The *B. burgdorferi* s.l. prevalence found in this study is similar to that reported from the Lista Bird Observatory in southern Norway, where *B. burgdorferi* s.l was detected in 5.4% and 7.5% of *I. ricinus* nymphs removed from birds in the spring and autumn, respectively (Kjelland et al. 2010). Later studies from the southern Norwegian coast reported 13.6% prevalence of *Borrelia* spp. in bird-feeding nymphs (Hasle et al. 2011); the study was, however, performed during the spring migration period. A large-scale study from southeastern Sweden showed a 19.3% prevalence of *B. burgdorferi* s.l. in nymphs collected from birds (Comstedt et al. 2006). In contrast, data from Central Europe showed higher *B. burgdorferi* s.l. prevalence in bird-feeding ticks. In Thuringia, Germany, *B. burgdorferi* s.l. was detected in 35% of nymphs removed from birds (Kipp et al. 2006). Similar results were reported from Czech Republic (Dubska et al. 2009) and Switzerland (Poupon et al. 2006), where *Borrelia* spp. prevalence in bird-derived nymphs was 31.4%, and 34.6%, respectively.

Common blackbirds and thrushes serve as main reservoirs for *B. garinii* and *B. valaisiana* in Europe (Humair et al. 1998, Hanincova et al. 2003b, Taragel'ova et al. 2008). The results of our study also support the notion that birds of *Turdus* spp. may play an essential role in distribution of *B. burgdorferi* s.l. by ticks in Europe, as 8 of 11 ticks infected with *Borrelia* were removed from *T. merula*. Furthermore, sequences of *B. garinii* and *B. valaisiana* detected in our study were identical to those of *B. garinii* 20047 (Postic et al. 1994) and NE11 (Gern et al. 2010), and to sequences of *B. valaisiana* genotype I-214 (Derdakova et al. 2003), which are widely spread in Europe.

There are several studies reporting the presence of *B. afzelii* in bird-feeding ticks (Humair et al. 1998, Comstedt et al. 2006, Kipp et al. 2006, Poupon et al. 2006, Taragel'ova et al. 2008, Franke et al. 2010b), although *B. afzelii* is regarded to be a rodent-associated genospecies in Europe (Humair et al. 1999, Hanincova et al. 2003a). It has been previously shown that birds do not serve as adequate reservoirs for *B. afzelii,* and furthermore, that the uptake of avian blood initiates the elimination of *B. afzelii* in the tick (Kurtenbach et al. 2002). Because the *B. afzelii* DNA, detected in the current study, was recovered from nymphs feeding on great tits, we suggest that *B. afzelii* infection in these ticks was acquired at the larval stage from a rodent host. However detection of a mix of *B. afzelii* strains PGau and Tom1503 in 1 nymph and detection of strain PGau in another nymph removed from the same great tit may indicate transmission of *B. afzelii* PGau strain through co-feeding on a bird. It is theoretically possible that the nymph acquired a mix of both strains of *B. afzelii* from a rodent during the larval stage (Hu et al. 1997, Humair et al. 1999), but transmission through co-feeding would be a much more likely event than that 2 ticks independently acquired the same strain of *B. afzelii* and accidentally entered the same host.

The low prevalence (0.4%) of *A. phagocytophilum* in bird-feeding ticks in the present study corresponds to the data from other studies (Franke et al. 2010a, Palomar et al. 2012). It is also in correspondence with our previous studies that showed 0.9% prevalence of *A. phagocytophilum* in *I. ricinus* ticks collected from the vegetation in the area near Kabli and Pulgoja bird stations (Katargina et al. 2012). Because this microorganism was detected in an *I. ricinus* nymph feeding on *T. iliacus*, short-distance migrants of *Turdus* species might be considered as potential dispersers of this tick-borne pathogen from Fennoscandia to Central Europe.

Conclusions

This is the first study on the detection of tick-borne pathogens in ticks removed from passerines during southward

migration from Estonia. These data support the notion that migratory birds may be essential in the long-distance distribution and introduction to new foci of TBEV, *B. burgdorferi* s.l., and *A. phagocytophilum* by transporting infected ticks while migrating from Fennoscandia and Russia to South Africa and Central Europe. Further large-scale studies are needed to reveal the significance of certain avian species in the maintenance and competence as reservoirs for different tick-borne pathogens in Europe.

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Author Disclosure Statement

No competing financial interests exist.

References

Alekseev AN, Dubinina HV, Van De Pol I, Schouls LM. Identification of *Ehrlichia* spp. and *Borrelia burgdorferi* in *Ixodes* ticks in the Baltic regions of Russia. J Clin Microbiol 2001; 39:2237–2242.

Bjoersdorff A, Bergstrom S, Massung RF, Haemig PD, et al. *Ehrlichia*-infected ticks on migrating birds. Emerg Infect Dis 2001; 7:877–879.

Comstedt P, Bergstrom S, Olsen B, Garpmo U, et al. Migratory passerine birds as reservoirs of Lyme borreliosis in Europe. Emerg Infect Dis 2006; 12:1087–1095.

Courtney JW, Kostelnik LM, Zeidner NS, Massung RF. Multiplex real-time PCR for detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi*. J Clin Microbiol 2004; 42:3164–3168.

Daniels TJ, Battaly GR, Liveris D, Falco RC, et al. Avian reservoirs of the agent of human granulocytic ehrlichiosis? Emerg Infect Dis 2002; 8:1524–1525.

Derdakova M, Beati L, Pet'ko B, Stanko M, et al. Genetic variability within *Borrelia burgdorferi* sensu lato genospecies established by PCR-single-strand conformation polymorphism analysis of the rrfA-rrlB intergenic spacer in *Ixodes ricinus* ticks from the Czech Republic. Appl Environ Microbiol 2003; 69:509–516.

Dubska L, Literak I, Kocianova E, Taragelova V, et al. Differential role of passerine birds in distribution of *Borrelia* spirochetes, based on data from ticks collected from birds during the postbreeding migration period in Central Europe. Appl Environ Microbiol 2009; 75:596–602.

Franke J, Meier F, Moldenhauer A, Straube E, et al. Established and emerging pathogens in *Ixodes ricinus* ticks collected from birds on a conservation island in the Baltic Sea. Med Vet Entomol 2010a; 24:425–432.

Franke J, Moldenhauer A, Hildebrandt A, Dorn W. Are birds reservoir hosts for *Borrelia afzelii*? Ticks Tick Borne Dis 2010b; 1:109–112.

Georgopoulou I, Tsiouris V. The potential role of migratory birds in the transmission of zoonoses. Vet Ital 2008; 44:671–677.

Gern L, Douet V, Lopez Z, Rais O, et al. Diversity of *Borrelia* genospecies in *Ixodes ricinus* ticks in a Lyme borreliosis endemic area in Switzerland identified by using new probes for reverse line blotting. Ticks Tick Borne Dis 2010; 1:23–29.

Golovljova I, Vene S, Sjolander KB, Vasilenko V, et al. Characterization of tick-borne encephalitis virus from Estonia. J Med Virol 2004; 74:580–588.

Gylfe A, Bergstrom S, Lundstrom J, Olsen B. Reactivation of *Borrelia* infection in birds. Nature 2000; 403:724–725.

Hanincova K, Schafer SM, Etti S, Sewell HS, et al. Association of *Borrelia afzelii* with rodents in Europe. Parasitology 2003a; 126:11–20.

Hanincova K, Taragelova V, Koci J, Schafer SM, et al. Association of *Borrelia garinii* and *B. valaisiana* with songbirds in Slovakia. Appl Environ Microbiol 2003b; 69:2825–2830.

Hasle G, Bjune GA, Midthjell L, Roed KH, et al. Transport of *Ixodes ricinus* infected with *Borrelia* species to Norway by northward-migrating passerine birds. Ticks Tick Borne Dis 2011; 2:37–43.

Hu CM, Humair PF, Wallich R, Gern L. *Apodemus* sp. rodents, reservoir hosts for *Borrelia afzelii* in an endemic area in Switzerland. Zentralbl Bakteriol 1997; 285:558–564.

Humair PF, Postic D, Wallich R, Gern L. An avian reservoir (*Turdus merula*) of the Lyme borreliosis spirochetes. Zentralbl Bakteriol 1998; 287:521–538.

Humair PF, Rais O, Gern L. Transmission of *Borrelia afzelii* from *Apodemus* mice and *Clethrionomys* voles to *Ixodes ricinus* ticks: Differential transmission pattern and overwintering maintenance. Parasitology 1999; 118(Pt 1):33–42.

Jaaskelainen AE, Sironen T, Murueva GB, Subbotina N, et al. Tick-borne encephalitis virus in ticks in Finland, Russian Karelia and Buryatia. J Gen Virol 2010; 91:2706–2712.

Katargina O, Geller J, Alekseev A, Dubinina H, et al. Identification of *Anaplasma phagocytophilum* in tick populations in Estonia, the European part of Russia and Belarus. Clin Microbiol Infect 2012; 18:40–46.

Kipp S, Goedecke A, Dorn W, Wilske B, et al. Role of birds in Thuringia, Germany, in the natural cycle of *Borrelia burgdorferi* sensu lato, the Lyme disease spirochaete. Int J Med Microbiol 2006; 296(Suppl 40):125–128.

Kjelland V, Stuen S, Skarpaas T, Slettan A. *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks collected from migratory birds in Southern Norway. Acta Vet Scand 2010; 52:59.

Kurtenbach K, Schafer SM, Sewell HS, Peacey M, et al. Differential survival of Lyme borreliosis spirochetes in ticks that feed on birds. Infect Immun 2002; 70:5893–5895.

Lommano E, Burri C, Maeder G, Guerne M, et al. Prevalence and genotyping of tick-borne encephalitis virus in questing *Ixodes ricinus* ticks in a new endemic area in western Switzerland. J Med Entomol 2012; 49:156–164.

Olsen B, Jaenson TG, Bergstrom S. Prevalence of *Borrelia burgdorferi* sensu lato-infected ticks on migrating birds. Appl Environ Microbiol 1995; 61:3082–3087.

Palomar AM, Santibanez P, Mazuelas D, Roncero L, et al. Role of birds in dispersal of etiologic agents of tick-borne zoonoses, Spain, 2009. Emerg Infect Dis 2012; 18:1188–1191.

Postic D, Assous MV, Grimont PA, Baranton G. Diversity of *Borrelia burgdorferi* sensu lato evidenced by restriction fragment length polymorphism of rrf (5S)-rrl (23S) intergenic spacer amplicons. Int J Syst Bacteriol 1994; 44:743–752.

Poupon M, Lommano E, Humair PF, Douet V, et al. Prevalence of *Borrelia burgdorferi* sensu lato in ticks collected from migratory birds in Switzerland. Appl Environ Microbiol 2006; 72:976–979.

Rar VA, Fomenko NV, Dobrotvorsky AK, Livanova NN, et al. Tickborne pathogen detection, Western Siberia, Russia. Emerg Infect Dis 2005; 11:1708–1715.

Schwaiger M, Cassinotti P. Development of a quantitative real-time RT-PCR assay with internal control for the laboratory

detection of tick borne encephalitis virus (TBEV) RNA. J Clin Virol 2003; 27:136–145.

Skarpaas T, Golovljova I, Vene S, Ljostad U, et al. Tickborne encephalitis virus, Norway and Denmark. Emerg Infect Dis 2006; 12:1136–1138.

Suss J. Tick-borne encephalitis 2010: Epidemiology, risk areas, and virus strains in Europe and Asia-an overview. Ticks Tick Borne Dis 2011; 2:2–15.

Taragel'ova V, Koci J, Hanincova K, Kurtenbach K, et al. Blackbirds and song thrushes constitute a key reservoir of *Borrelia garinii*, the causative agent of borreliosis in Central Europe. Appl Environ Microbiol 2008; 74:1289–1293.

Waldenstrom J, Lundkvist A, Falk KI, Garpmo U, et al. Migrating birds and tickborne encephalitis virus. Emerg Infect Dis 2007; 13:1215–1218.

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PUBLICATION II

Detection and genetic characterization of relapsing fever spirochete *Borrelia miyamotoi* in Estonian ticks

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Detection and Genetic Characterization of Relapsing Fever Spirochete *Borrelia miyamotoi* in Estonian Ticks

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Abstract

During the years 2008–2010 *I. ricinus* and *I. persulcatus* ticks were collected from 64 sites in mainland Estonia and on the island Saaremaa. Presence of *B. miyamotoi* was found in 0.9% (23/2622) of ticks. The prevalence in *I. persulcatus* and *I. ricinus* ticks differed significantly, 2.7% (15/561) and 0.4% (8/2061), respectively. The highest prevalence rates were in found South-Eastern Estonia in an area of *I. persulcatus* and *I. ricinus* sympatry and varied from 1.4% (1/73) to 2.8% (5/178). Co-infections with *B. burgdorferi s.l.* group spirochetes and tick-borne encephalitis virus were also revealed. Genetic characterization of partial 16S rRNA, p66 and glpQ genes demonstrated that Estonian sequences belong to two types of *B. miyamotoi* and cluster with sequences from Europe and the European part of Russia, as well as with sequences from Siberia, Asia and Japan, here designated as European and Asian types, respectively. Estonian sequences of the European type were obtained from *I. ricinus* ticks only, whereas the Asian type of *B. miyamotoi* was shown for both tick species in the sympatric regions.

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Introduction

The *Borrelia* genus consists of two groups of species [1].The Lyme borreliosis (LB) group of spirochetes include agents that cause disease (LB) in humans as well as some species not associated with human disease. The LB group organisms are widely spread in Europe and North America and transmitted between vertebrates by hard (ixodid) ticks [2]. The relapsing fever (RF) group spirochetes mainly use soft (argasid) ticks as vectors [3] but some of them are transmitted also by hard tick vectors. This group includes *B. theileri*, which is vectored by *Rhipicephalus* ticks and causes infections in large livestock, *B. lonestari*, which is transmitted by *Ambylomma americanum* and causes infections in deer [4], as well as *B. miyamotoi*, which is transmitted by *Ixodes* ticks and is found in a small percentage of ticks in Eurasia and North America [5,6,7,8,9].

B. miyamotoi was isolated for the first time in Japan in 1995 from *I. persulcatus* ticks as well as from blood of *Apodemus argenteus* mice [7,10]. DNA of closely related spirochetes was subsequently detected in *I. scapularis* [11] and *I. pacificus* [12] in the USA. In Europe, *B. miyamotoi* was detected in *I.ricinus* ticks in Sweden [6] and Germany [9]. In European and Asian regions of Russia DNA of *B. miyamotoi* was detected in *I.persulcatus* [13] and *I.ricinus* as well as in human blood [8]. In addition to *A. argenteus*, it has been shown that white-footed mice (*Peromyscus leucopus*) may serve as host reservoirs for *B. miyamotoi* [11] and detection of *B. miyamotoi* from wild turkeys (*Meleagris gallopavo*) was also recently reported [14].

Unlike LB spirochetes, *B. miyamotoi* and other relapsing fever spirochetes are vertically transmittable from a female adult tick to her offspring [15,16,17]. Also transmission of spirochetes by co-feeding from nymph to larvae and horizontal transmission from infected mice to ticks was experimentally shown [4,12], although at a lower rate compared to *B. burgdorferi* s.l. [11].

Over the last decade *B. miyamotoi* has been detected in *Ixodes* ticks in the USA [11,12], Sweden [18], Czech Republic [16], France, and Germany [9] as well as in Russia [13,19,20]. Human disease caused by this RF group spirochete has not been well characterized, but recently probable cases of *B. miyamotoi* infection in RF-patients were reported from Russia [8,21,22].

Our aim was to investigate the presence and the prevalence of *B. miyamotoi* in different areas of Estonia.

Materials and Methods

Ethics Statement

According to Estonian legislation no specific permits were required for the described field studies. None of the locations described in the study were situated on the private land, in the National parks nor protected area. The described field studies did not involve endangered or protected species.

Collection of Ticks

Ticks were collected from the vegetation by flagging from April to November during 2008–2010 at 64 sites in mainland Estonia and on Saaremaa island (Figure 1, Table 1). Tick species were

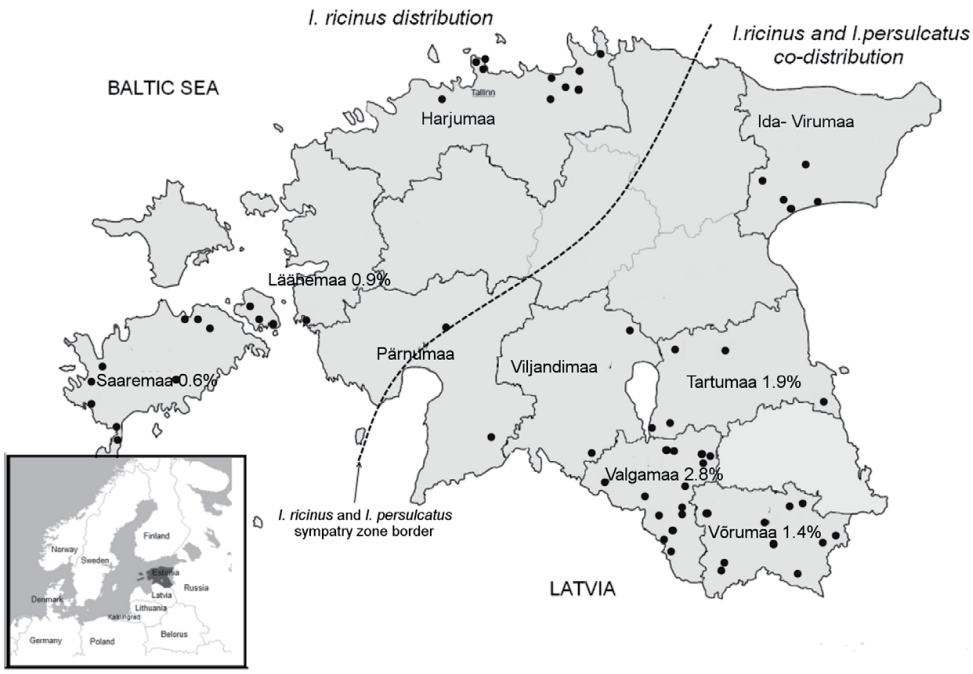

Figure 1. Tick sampling sites in Estonia and the prevalence of *B. miyamotoi*. The areas of *I. ricinus* and *I. persulcatus* distribution and sympatry are indicated. The ticks' collection sites indicated by dots.
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independently identified by morphological criteria by two entomologists, washed in 70% ethanol, rinse twice with sterile PBS and individually stored at −70°C.

Extraction of DNA

Ticks were homogenized in 300 µl of PBS by TissueLyser (Retsch, Haan, Germany). Two hundred microliters of suspensions were used for DNA extraction. DNA was extracted by the guanidinium thiocyanate-phenolchloroform method using the TriPure isolation system (Roche Diagnostics, Lewes, UK) according to the manufacturer's recommendations. Sterile water was included as a negative control for every DNA preparation set.

Statistics

Fisher's exact and Poisson probability tests were used to assess differences in *B. miyamotoi* prevalence in *I. ricinus* and *I. persulcatus* ticks.

Detection of *B. miyamotoi, B. burgdorferi* s.l. and Tick-borne Encephalitis Virus in Ticks

The presence of Borrelia species was detected by amplification of 1256 bp product of 16S rRNA partial gene as described [7] with external primers 16S-Bor-S1F and 16S-Bor-S2R under the following conditions: 35 cycles, 94°C-10 sec, 60°C-1 min, 72°C-90 sec. Nested PCR was performed with primer pair 16S-Bor-S4F and 16S-Bor-S3R [13] and cycling conditions included 35 cycles of initial denaturation at 94°C for10 sec, annealing at 65°C-1 min, elongation at 72°C-90 sec.

To distinguish *B. miyamotoi* from *B. burgdorferi* s.l. among all 16S PCR- positive samples primers targeted *B. miyamotoi* p66 gene were chosen. The amplification of p66 partial gene was performed as described previously [13] with external primers pair M1F and M2R at the following conditions: 35 cycles, 94°C-5 sec, 50°C-10 sec, 72°C-30 sec. A 532 bp product was generated using inner primers M3F and M4R. The annealing time was increased to 15 sec and elongation time to 45 sec.

All p66-positive samples were further used for glpQ partial gene amplification as described by Fomenko et al [13]. Primers Q1F and Q2R were used for the first round of PCR, and cycling conditions included 35 cycles, 94°C–10 sec, 50°C–15 sec, 72°C–35 sec. Inner primers Q3F and Q4R were used in a nested PCR for generation of a 379 bp product at the following cycling conditions: 35 cycles, 94°C–5 sec, 52°C–10 sec, 72°C–30 sec.

To reveal co-infections of ticks with *B. miyamotoi* and *B. burgdorferi* s.l., samples positive for *B. miyamotoi* were amplified by nested PCR for *B. burgdorferi* s.l.-group specific 5S-23S rRNA intergenic spacer (IGS) region as described previously [23,24] with a modified touch-down program. The first amplification round included 35 cycles, 94°C–1 min, 58°C–1 min and 72°C–2 min, and in the nested PCR, the annealing temperature was decreased to 52°C and amplification was performed for 30 cycles.

Tick-borne encephalitis virus (TBEV) detection was performed by PCR amplification and further sequencing of partial E gene as described earlier [25] with outer primers 283F1 and 827R1 used for the cDNA synthesis and inner primers 349F2 and 814R2 for the second round of PCR amplification.

To confirm the morphological tick species definition, *B. miyamotoi* positive samples were analyzed for mitochondrial 16S

Table 1. *Borrelia miyamotoi* detection in ticks and estimated prevalence (%).

Place of collection	*I. ricinus*			*I. persulcatus*			Total no. ticks infected/tested (%)	
	No. adults infected/tested (%)	No. nymphs infected/tested (%)	Total no. ticks infected/tested (%)	No. adults infected/tested (%)	No. nymphs infected/tested (%)	Total no. ticks infected/tested (%)		
Ida-Virumaa	0/43	0/19	0/62	0/92	0/9	0/101	0/163	*I. persulcatus* and *I. ricinus* sympatric area 17/1324 (1.3%)[§]
Viljandimaa	0/2	–*	0/2	0/44	–	0/44	0/46	
Tartumaa	0/226	2/66 (3.0%)	2/292 (0.7%)	5/187 (2.7%)	4/94 (4.3%)	9/281 (3.2%)	11/573 (1.9%)	
Valgamaa	0/57	–	0/57	5/121 (4.1%)	–	5/121 (4.1%)	5/178 (2.8%)	
Võrumaa	0/64	–	0/64	1/9 (11.1%)	–	1/9 (11.1%)	1/73 (1.4%)	
Pärnumaa	0/180	0/106	0/286	0/4	0/1	0/5	0/291	
Läänemaa	0/97	1/10 (10%)	1/107 (0.9%)	–	–	–	1/107 (0.9%)	*I. ricinus* area 6/1298 (0.5%)[§]
Harjumaa	0/217	0/77	0/294	–	–	–	0/294	
Saaremaa	4/508 (0.8%)	1/389 (0.3%)	5/897 (0.6%)	–	–	–	5/897 (0.6%)	
Total	4/1394 (0.3%)	4/667 (0.6%)	8/2061 (0.4%)[‡]	11/456 (2.4%)	4/104 (3.8%)	15/561 (2.7%)[‡]	23/2622 (0.9%)	

*Not collected.

[‡]$P<0.0001$ Fisher's exact and Poisson probability tests.

[§]$P<0.05$ Fisher's exact test; $P<0.001$ Poisson probability test.

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rRNA partial gene PCR as described by Caporale et al [26] with further sequencing using primers 16Sa and 16Sb. Cycling conditions included pre-PCR steps 95°C–1 min; 49°C–1 min, 72°C–2 min and 95°C–1 min; 47°C–1 min; 72°C–2 min and amplification 40 cycles, 95°C–30 sec; 45°C–1 min; 72°C–2 min.

PCR products were visualized by electrophoresis in 1% agarose gel stained with ethidium bromide. Deionized water was included in every PCR step as a negative control.

To minimize contamination, reaction mix preparation, sample addition step, amplification and gel electrophoresis were performed in three separate rooms with sterile techniques. Sample addition was performed in a laminar flow cabinet.

Phylogenetic Analysis

Analysis and alignment of sequences was performed using BioEdit 7.0.0 software. The Maximum Likelihood model was used for phylogenetic tree reconstruction of the partial 16S rRNA (1106 bp), p66 (349 bp and 355 bp for "*I. persulcatus*"-type and "*I. ricinus*"-type, respectively) and glpQ genes (379 bp), using the Tree Puzzle program. 10 000 puzzling steps were applied using the GTP model of substitution for the partial 16S rRNA gene and the Hasegawa-Kishino-Yano (HKY) model for the partial p66 and glpQ genes.

Results

Tick Collections and Detection of *B. miyamotoi* DNA

In total, 2622 ticks (1851 adults and 771 nymphs) were collected in 64 sites of 9 Estonian counties, and among them 2061 (78.6%) and 561 (21.4%) were identified as *I. ricinus* and *I. persulcatus*, respectively (Table 1). DNA of *B. miyamotoi* was detected in 23 (0.9%) tick suspensions, 15 of which originated from *I. persulcatus* and 8 from *I. ricinus* ticks. Thus the overall prevalence of *B. miyamotoi* in *I. persulcatus* ticks was 2.7% (15/561) and 0.4% (8/2061) in *I. ricinus* ticks ($P<0.0001$, Fisher's exact and Poisson probability tests). The highest prevalence of *B. miyamotoi* in tick populations was detected in the South-Eastern Estonia, in Valgamaa (2.8%), Tartumaa (1.9%) and Võrumaa (1.4%) counties. This region is sympatric for both tick species and DNA of *B. miyamotoi* was found mainly in *I. persulcatus*. However, in other sympatric areas, Ida-Virumaa, Viljandimaa and Pärnumaa, *B. miyamotoi* was not detected. In regions where only *I. ricinus* circulates, the prevalence of *B. miyamotoi* was lower –0.9% and 0.6% in Läänemaa and on Saaremaa island, respectively. Comparison of the prevalence rates of *B. miyamotoi* in areas sympatric for both tick species and areas where only *I. ricinus* circulates demonstrated a statistically significant difference, 1.3% vs 0.5% ($P<0.05$ Fisher's exact test and $P<0.001$ Poisson probability test).

In our study we did not find differences in *B. miyamotoi* prevalence between different tick stages, as *B. miyamotoi* DNA was detected in 1% of nymphal ticks (8 out of 771) and in 0.8% of adult ticks (15 out of 1851).

Co-infection with spirochetes belonging to *Borrelia burgdorferi* s.l. was demonstrated by amplification of 5S-23S IGS, which is specific for the *B. burgdorferi* s.l. group. We showed that 5 ticks (21.7% from all positive ticks) were co-infected with *B. afzelii*, *B. garinii* or *B. valaisiana* (Table 2). Co-infection with another widely distributed tick-borne pathogen, TBEV, was found in adult *I. ricinus* ticks on Saaremaa island. Genetic analysis of the partial E gene sequence revealed that this strain belonged to the European subtype of TBEV.

Genetic and Phylogenetic Analysis of *B. miyamotoi* Sequences

Three genomic regions of *B. miyamotoi*, the partial p66 (532 bp), 16S rRNA (1256 bp) and qlpQ (379 bp) genes, were sequenced for genetic characterization of Estonian samples. Fourteen tick suspensions were amplified for all three genes, 6 for two genes and 3 for one gene region. Analysis of nucleotide sequence similarity of the three genomic regions showed that Estonian samples were divided into two groups: the first with sequences identical to those amplified from *I. ricinus* in Sweden and the European part of Russia (European type) and the second with sequences identical to those found in *I. persulcatus* and human blood in the European part of Russia, Ural and Siberia (Asian type). Within each group, the sequences of the Estonian samples were identical for all three gene regions. Moreover, in the European type cluster, sequences of the partial 16S rRNA, p66 and qlpQ genes amplified in the present study were identical to the *B. miyamotoi* sequences derived from GenBank and detected in Sweden, the European part of Russia, Poland, and France. In the Asian type cluster, the Estonian sequences were identical to those amplified from ticks from different parts of Russia (European part, Ural, Siberia) and also Japan, with the exception of D45192 and AF228023 for the partial 16S rRNA and p66 genes, respectively.

Nucleotide sequence identity between European type and Asian type groups were found 99.4–99.6% for the partial 16S rRNA gene, 98.6% for the partial glpQ gene and 91.6–93.3% for the partial p66 gene. Nucleotide sequences of the partial p66 gene were more diverse and insertion of 6 nucleotides was demonstrated for European type of *B. miyamotoi* when compared to the Asian type.

On the phylogenetic trees based of the partial p66, partial 16S rRNA and qlpQ genes (Figure 2) sequences detected in the present study clustered with *B. miyamotoi* sequences detected in Siberia and Japan as well as with sequences from Europe and the European part of Russia. Estonian sequences together with previously reported sequences of *B. miyamotoi* formed well supported European type and Asian type clusters. Closely related sequences of *B. miyamotoi* amplified from *I. scapularis* in USA clustered together with the European type of sequences in the phylogenetic tree based on the partial p66 gene sequences, while on tree based on the partial 16S rRNA gene it formed its own lineage albeit with a low bootstrap support.

The two groups of *B. miyamotoi* correspond to the tick species from which sequences were amplified: European type sequences were amplified from *I. ricinus* in Europe and Asian type from *I. persulcatus* in Japan, Siberia and Ural, and additional sequences belonging to this type were detected in blood of patients in Siberia [21] and *A. argenteus* in Japan [7,10]. In the current study we found the Asian type of *B. miyamotoi* in two *I. ricinus* nymphs (Est3489-2 and Est3115-1) in an area sympatric for both tick species (Tartumaa). Species identification of these nymphs as *I. ricinus* by morphological criteria was confirmed by sequencing of the partial mitochondrial 16S rRNA gene.

Discussion

In the current study, DNA of relapsing fever spirochetes of *B. miyamotoi* was for the first time detected in ticks in Estonia. We found statistically significant differences between the prevalence rates of *B. miyamotoi* DNA in *I. persulcatus* and *I. ricinus* ticks, 2.7% and 0.4%, respectively. Similar prevalence rates were reported from a sympatric region, Moscow province, at 1.5% in *I. persulcatus* and 0.6% in *I. ricinus* [8]. Previously published data of *B. miyamotoi* DNA detection in *I. persulcatus* demonstrated 2.3–4.5% prevalence

Table 2. *B. miyamotoi* infections in Estonian ticks.

	Place of collection	Species of tick		Type of *B. miyamotoi*	Co-infection with other TBP*
Est1868	Tartumaa	*I. persulcatus*	F	Asian	
Est1885		*I. persulcatus*	F	Asian	
Est3943-4		*I. persulcatus*	F	Asian	
Est1811		*I. persulcatus*	N†	Asian	
Est3486-4		*I. persulcatus*	F	Asian	
Est3487-4		*I. persulcatus*	N	Asian	
Est3698-2		*I. persulcatus*	N	Asian	
Est722-2		*I. persulcatus*	N	Asian	
Est1586		*I. persulcatus*	N	Asian	*B. valaisiana*
Est3115-1		*I. ricinus*	N	Asian	
Est3489-2		*I. ricinus*	F	Asian	
Est4318	Valgamaa	*I. persulcatus*	M	Asian	
Est4350		*I. persulcatus*	F	Asian	
Est4372		*I. persulcatus*	M	Asian	*B. afzelii* (VS461 group)
Est4243		*I. persulcatus*	F	Asian	
Est4412		*I. persulcatus*	F	Asian	*B. garinii* (NT29 group)
Est3633	Võrumaa	*I. persulcatus*	M	Asian	*B. afzelii* (VS461 group)
Est2519	Saaremaa	*I. ricinus*	F	European	
Est3849		*I. ricinus*	M	European	
Est2270		*I. ricinus*	M	European	TBEV-Eu subtype
Est2409		*I. ricinus*	M	European	*B. garinii* (20047 group)
Est2325-3		*I. ricinus*	N	European	
Est1129-4	Läänemaa	*I. ricinus*	N	European	

*Tick-borne pathogen.
†Nymph.
doi:10.1371/journal.pone.0051914.t002

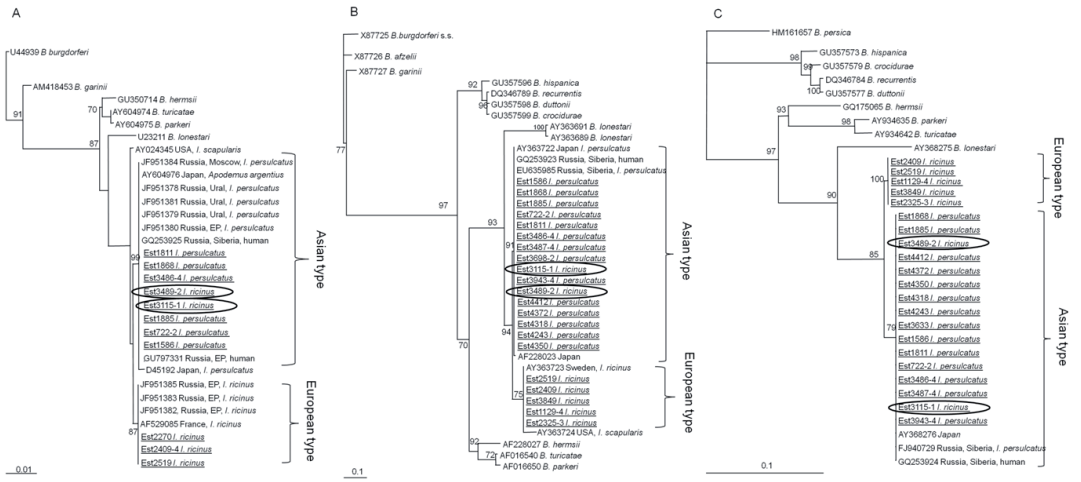

Figure 2. Phylogenetic trees based on the partial sequences of 16S rRNA, p66 and glpQ genes. The Maximum Likelihood model was used for phylogenetic tree reconstruction of the partial A) 16S rRNA (1106 bp), B) p66 (349 bp and 355 bp for ''*I. persulcatus*''-type and ''*I. ricinus*''-type, respectively) and C) glpQ genes (379 bp). Only quartet puzzling support values >70% are shown. Samples sequenced in the present study are underlined.
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in Siberia [13,19] and 0.9%–16% in Ural [8]. The reported prevalence of *B. miyamotoi* DNA in *I. ricinus* ticks fluctuated from 0.5% in the European part of Russia [8] to 3.5% in Germany [9]. In the USA, similar *B. miyamotoi* prevalence rates in ticks were reported and correspond with those found in Europe: 1.9–2.5% to 6% for *I. scapularis* [11] and 1.7% and 0.7% for *I. pacificus* nymphs and adults, respectively [12]. In all studies where *B. miyamotoi* and *Borrelia* species belonging to LB complex were simultaneously detected, the prevalence rates of *B. miyamotoi* were significantly lower than those of *B. burgdorferi* s.l. [4,9,13,19].

In the current study we found statistically significant differences of *B. miyamotoi* prevalence rates in ticks between the region of *I. ricinus* range (0.5%) and the region sympatric for both tick species (1.3%). This fact may be explained either by a higher tropism of *B. miyamotoi* to *I. persulcatus* ticks or by more favorable conditions for pathogen circulation (microclimate, abundance of small and big mammals and etc.) in sympatric area in Eastern Estonia. However, simultaneous detection of *B. miyamotoi* DNA in both species of ticks collected in the sympatric area, although less frequently in *I. ricinus* compared to *I. persulcatus,* allows us to suggest that the latter explanation is the more probable one. Our investigation of tick-borne encephalitis virus and *Borrelia burgdorferi* s.l. prevalence in ticks demonstrated similar results, with statistically significant differences of prevalence rates between Western and Northern Estonia (areas of *I. ricinus* circulation) and Eastern Estonia (sympatric area for *I. persulcatus* and *I. ricinus*). However, statistically significant differences were not found between the two tick species in the sympatric area (our unpublished data).

In the current study we did not find statistically significant difference in the prevalence of *B. miyamotoi* in adults (0.8%) and nymphs (1%); observations that correspond to findings in the American ticks *I. pacificus* and *I. scapularis* [4,12] as well as in European *I. ricinus* ticks [9]. Analysis of larval tick stages should be performed for accurate assessment of a cumulative risk of *B. miyamotoi* infection with each subsequent feeding.

In the present study, 21.7% of *B. miyamotoi* positive ticks were also co-infected with spirochetes of the *B. burgdorferi* s.l. genospecies, and in one case a co-infection with TBEV was found. Thus in Estonia *B. miyamotoi* and Lyme disease spirochetes may share hosts, which is in contrast to findings in Germany and France [9] and the USA [4,11]. Moreover, it has recently been reported that *B. burgdorferi* and *B. miyamotoi* circulate among a separate set of hosts and utilize different transmission loops: for *B. burgdorferi* it is exclusively transmission to susceptible larvae feeding on hosts previously infected by nymphs, while *B. miyamotoi* utilizes mix of vertical and horizontal transmission in the Midwest of the USA [15]. However, *B. miyamotoi* co-infections with *B. garinii* (2.8%) and *B. afzelii* (0.2%) have also been reported in *I. persulcatus* ticks in Siberia [19]. Thus we may suggest that co-feeding on the same host and consequently co-infections of relapsing fever and Lyme disease spirochetes depend on local climatic and environmental conditions and could occur in Estonia and Siberia.

Genetic and phylogenetic analysis of the three gene regions of *B. miyamotoi* revealed that the Estonian sequences divided into two groups, the European and Asian groups, respectively, and that within each group the sequences were identical or shared a high level of similarity in a very large geographical range from Northern Europe (Sweden, Estonia) to the European part of Russia for the first group, and from Estonia, the European part of Russia to Siberia and Japan for the second group. The European type of *B. miyamotoi* sequences have been detected in *I. ricinus* ticks while the Asian has been found in *I. persulcatus* ticks and human blood [5,6,7,8,13,27]. In the present study we found that the Asian type of *B. miyamotoi* may be exchanged between tick species in a sympatric area, although not at a very high rate: among 16 sequences of the Asian group, 14 were amplified from *I. persulcatus* and two from *I. ricinus*. Similar results we found for TBEV, sequences belonging to the Siberian subtype of TBEV (TBEV-Sib), which were detected not only in *I. persulcatus* (the natural vector of TBEV-Sib) but also in *I. ricinus* collected in the same sympatric area (our unpublished data).

Recently it has been reported that *B. miyamotoi* probably causes relapsing fever (RF) and Lyme disease-like symptoms in Ural [8] and Siberia [21], and all the reported sequences from patients belonged to the Asian group of *B. miyamotoi*. In Estonia and other parts of Europe human cases of RF caused by *B. miyamotoi* infection have to date not been reported. The reason remains unclear; it may be either underreporting of *B. miyamotoi* infection due to serological cross-reactions in ELISA with *B. burgdorferi* s.l. antigens or a different pathogenecity of the European lineage of *B. miyamotoi*. Further investigations need to be performed in order to understand the vector potential of *I. ricinus* ticks for the Asian lineage of *B. miyamotoi*, which may be useful for the prediction of a possible spread of this group of spirochetes in a westward direction into Europe.

Author Contributions

Conceived and designed the experiments: IG NF JG. Performed the experiments: JG LN OK IG. Analyzed the data: IG JG LJ NF OK. Contributed reagents/materials/analysis tools: IG LJ. Wrote the paper: IG JG OK LN NF LJ.

References

1. Paster BJ, Dewhirst FE, Weisburg WG, Tordoff LA, Fraser GJ, et al. (1991) Phylogenetic analysis of the spirochetes. J Bacteriol 173: 6101–6109.
2. Steere AC.; Coburn JG, L. (2005) Lyme borreliosis. In: Goodman JD, D; Sonenshine, DE, editor. Tick-Borne Diseases of Humans. Washington, DC: ASM Press. 176–206.
3. Barbour A (2005) Relapsing fever. In: Goodman JD, D; Sonenshine, DE. editor. Tick-Borne Diseases of Humans. Washington, DC: ASM Press. 268–291.
4. Barbour AG, Bunikis J, Travinsky B, Hoen AG, Diuk-Wasser MA, et al. (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.
5. Bunikis J, Tsao J, Garpmo U, Berglund J, Fish D, et al. (2004) Typing of *Borrelia* relapsing fever group strains. Emerg Infect Dis 10: 1661–1664.
6. Fraenkel CJ, Garpmo U, Berglund J (2002) Determination of novel *Borrelia* genospecies in Swedish *Ixodes ricinus* ticks. J Clin Microbiol 40: 3308–3312.
7. Fukunaga M, Takahashi Y, Tsuruta Y, Matsushita O, Ralph D, et al. (1995) Genetic and phenotypic analysis of *Borrelia miyamotoi* sp. nov., isolated from the ixodid tick *Ixodes persulcatus*, the vector for Lyme disease in Japan. Int J Syst Bacteriol 45: 804–810.
8. Platonov AE, Karan LS, Kolyasnikova NM, Makhneva NA, Toporkova MG, et al. (2011) Humans infected with relapsing fever spirochete *Borrelia miyamotoi*, Russia. Emerg Infect Dis 17: 1816–1823.
9. Richter D, Schlee DB, Matuschka FR (2003) Relapsing fever-like spirochetes infecting European vector tick of Lyme disease agent. Emerg Infect Dis 9: 697–701.
10. 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.
11. Scoles GA, Papero M, Beati L, Fish D (2001) A relapsing fever group spirochete transmitted by *Ixodes scapularis* ticks. Vector Borne Zoonotic Dis 1: 21–34.
12. Mun J, Eisen RJ, Eisen L, Lane RS (2006) Detection of a *Borrelia miyamotoi* sensu lato relapsing-fever group spirochete from *Ixodes pacificus* in California. J Med Entomol 43: 120–123.
13. Fomenko NV, Livanova NN, Borgoiakov V, Kozlova IV, Shulaikina IV, et al. (2010) [Detection of *Borrelia miyamo*toi in ticks *Ixodes persulcatus* from Russia]. Parazitologiia 44: 201–211.
14. Scott MC, Rosen ME, Hamer SA, Baker E, Edwards H, et al. (2010) High-prevalence *Borrelia miyamotoi* infection among [corrected] wild turkeys (*Meleagris gallopavo*) in Tennessee. J Med Entomol 47: 1238–1242.

15. Davis S, Bent SJ (2011) Loop analysis for pathogens: niche partitioning in the transmission graph for pathogens of the North American tick *Ixodes scapularis*. J Theor Biol 269: 96–103.
16. Richter D, Debski A, Hubalek Z, Matuschka FR (2012) Absence of Lyme disease spirochetes in larval *Ixodes ricinus* ticks. Vector Borne Zoonotic Dis 12: 21–27.
17. Schwan TG, Piesman J (2002) Vector interactions and molecular adaptations of lyme disease and relapsing fever spirochetes associated with transmission by ticks. Emerg Infect Dis 8: 115–121.
18. Wilhelmsson P, Fryland L, Borjesson S, Nordgren J, Bergstrom S, et al. (2010) Prevalence and diversity of *Borrelia* species in ticks that have bitten humans in Sweden. J Clin Microbiol 48: 4169–4176.
19. Borgoiakov V, Fomenko NV, Panov VV, Chikova ED (2011) [Study on the infection of taiga ticks with *Borrelia* in the territory of Novosibirsk Scientific Center SB PAS]. Parazitologiia 44: 543–556.
20. Korotkov Y S, Kislenko GS, Burenkova LA, Rudnikova NA, Karan LS (2008) [Spatial and temporal variability of *Ixodes ricinus* and *Ixodes persulcatus* infection with the Lyme disease agent in Moscow Region]. Parazitologiia 42: 441–451.
21. Fomenko NV, Epikhina TI, Chernousova NY (2010) Detection of *Borrelia miyamotoi* in the blood of patients got disease in the spring-summer epidemiological period. Molekuliarnaya meditsina: 28–31.
22. Karan LS, Koliasnikova NM, Toporkova MG, Makhneva MA, Nadezhdina MV, et al. (2010) [Usage of real time polymerase chain reaction for diagnostics of different tick-borne infections]. Zh Mikrobiol Epidemiol Immunobiol: 72–77.
23. Postic D, Assous MV, Grimont PA, 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.
24. Rar VA, Fomenko NV, Dobrotvorsky AK, Livanova NN, Rudakova SA, et al. (2005) Tickborne pathogen detection, Western Siberia, Russia. Emerg Infect Dis 11: 1708–1715.
25. Skarpaas T, Golovljova I, Vene S, Ljostad U, Sjursen H, et al. (2006) Tickborne encephalitis virus, Norway and Denmark. Emerg Infect Dis 12: 1136–1138.
26. Caporale DA, Rich SM, Spielman A, Telford SR, 3rd, Kocher TD (1995) Discriminating between *Ixodes* ticks by means of mitochondrial DNA sequences. Mol Phylogenet Evol 4: 361–365.
27. Fomenko NV, Borgoiakov V, Panov VV (2011) [Genetic features of *Borrelia miyamotoi* transmitted by Ixodes persulcatus]. Mol Gen Mikrobiol Virusol: 12–17.

PUBLICATION III

Borrelia burgdorferi sensu lato prevalence in tick populations in Estonia

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Borrelia burgdorferi sensu lato prevalence in tick populations in Estonia

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Abstract

Background: Estonia is located in a unique area of co-distribution of *Ixodes ricinus* and *I. persulcatus*, which are the main tick vectors of *Borrelia burgdorferi* sensu lato. In the last decade, the incidence rate of Lyme borreliosis in Estonia has increased dramatically up to 115.4 per 100,000 in 2012. Here we present the first survey of the presence, the prevalence and genetic characteristics of *B. burgdorferi* s.l. complex spirochetes in the tick population in Estonia.

Methods: During the years 2006–2009, 2833 unfed *Ixodes ricinus* and *I. persulcatus* were collected from 43 sites in 7 counties in mainland Estonia as well as in 10 sites on the Saaremaa Island. DNA samples from ticks were analyzed individually using nested PCR of the ribosomal 5S-23S spacer region followed by bidirectional sequencing.

Results: The overall estimated prevalence of *B. burgdorferi* s.l was 9.7% and varied from 4.9% to 24.2% on the mainland and to 10.7% in Saaremaa Island. *Ixodes persulcatus* ticks showed significantly higher prevalence rates compared to that in *I. ricinus*-16.3% and 8.2%, respectively. The most prevalent genospecies was *B. afzelii* which was detected in 53.5% of *Borrelia*-positive ticks, followed by *B. garinii* and *B. valaisiana* with 26.2% and 5.5%, respectively. Also, *B. bavariensis* and *B. burgdorferi* s.s. DNA in single *I. ricinus* ticks were detected. *Borrelia afzelii*, *B. garinii* and *B. valaisiana* were detected in both tick species. Two genetic subgroups of *B. garinii* (NT29 and 20047) and two genetic subgroups of *B. afzelii* (NT28 and VS461) were found to be circulating in all studied regions as well as in both tick species, except *B. garinii* subgroup NT29, which was found only in *I. persulcatus* ticks.

Conclusions: In the current study we detected the circulation of five *B. burgdorferi* s.l. genospecies and estimated the prevalence in ticks in different regions of Estonia. Detection and genetic characterization of *Borrelia* genospecies, especially those of public health importance, in the natural foci may help assessing high risk areas of human exposure to *B. burgdorferi* s.l.

Keywords: Ticks, *Borrelia burgdorferi* sensu lato, Ixodes, Lyme disease

Background

Ticks are important vectors of human and animal pathogens of viral, bacterial and protozoan nature worldwide. Lyme borreliosis (LB) is the most widely spread and most frequent tick-borne bacterial disease in Europe with an estimated 65,500 human cases annually [1]. In Estonia the incidence rate of LB has increased in the last decade from 23.4 in 2002 to 115.4 per 100,000 in 2012 [2], which are the highest rates in the Baltic region [3].

The causative agents of LB are the members of *Borrelia burgdorferi* sensu lato complex spirochetes of which *B. afzelii*, *B. garinii*, *B. spielmanii* and *B. burgdorferi* sensu stricto are known to be pathogenic for humans, and *B. valaisiana*, and *B. lusitaniae* are considered potentially pathogenic [4]. The first five of these genospecies are widely spread in Europe with a predominance of *B. afzelii* and *B. garinii* [5]. It has been shown that rodents are the main reservoir for *B. afzelii* [6] and *B. bavariensis* [7], while *B. garinii* and *B. valaisiana* are associated mostly with birds [8,9] and *B. burgdorferi* s.s. circulates in both rodent and avian hosts [10].

Ticks of the *Ixodes* spp. are the main vectors for *Borrelia* spirochetes. *Ixodes ricinus* and *I. persulcatus* ticks are the main vectors of *B. burgdorferi* s.l. in the natural foci in Europe and Asia. Nymphs are the most important in the infection cycle, while the role of adults

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is insignificant, as male *Ixodes* ticks usually do not feed and female ticks prefer feeding on larger mammals that are usually not competent hosts for *Borrelia* spirochetes [11]. *Borrelia burgdorferi* s.l. is transmitted transstadially, while transovarial transmission of spirochetes from the female to her offspring is a rare event [5,12]. Larvae acquire *Borrelia* during their first bloodmeal on an infected competent reservoir hosts. After molting to nymphs they transmit the infection to new uninfected hosts while feeding. Effective transmission of *Borrelia* from infected to uninfected ticks via co-feeding has also been shown to occur without development of systemic infection in hosts [13,14].

Estonia is situated in the area of *I. ricinus* and *I. persulcatus* co-distribution providing a special setting in Eastern Europe for the study of tick-borne pathogens.

A previous report from Vormsi Island has shown the presence of *B. burgdorferi* s.l. genospecies in *I. ricinus* ticks [15]. The present study is the first survey of detection and genetic characterization of *B. burgdorferi* s.l. genospecies in both tick species in different regions in mainland Estonia as well as on the Saaremaa Island.

Methods

Tick collection

Ticks were collected at 43 sites in 8 counties in mainland Estonia and 10 sites on the largest island Saaremaa during the years 2006–2009. All study sites on the mainland were grouped into 7 regions according to their geographical and administrative location, whereas 4 regions with a total of 34 sites were located in the zone of *I. ricinus* and *I. persulcatus* sympatry (Figure 1).

Questing nymphs and adult ticks of both tick species were collected by passing a 1 m^2 flannel flag over the vegetation during the tick activity period from April until October in 2006–2009. The cloths were examined after every 5 m, all ticks were removed with forceps and maintained alive until later identification. Tick species, sex and stage were identified morphologically using a stereomicroscope. All ticks were investigated individually. Ticks were homogenized in 300 microliters of PBS with TissueLyzer (Haas, Germany). Two hundred microliters of suspension were used for DNA extraction and one hundred microliters were stored at −70°C.

DNA extraction

The guanidinium thiocyanate-phenol-chloroform method with TriPure isolation system (Roche Diagnostics, Lewes, UK) was used for DNA extraction according to the manufacturer's recommendations. Sterile water was included as a negative control for every DNA preparation set. DNA was kept at −20°C until further use.

PCR amplifications

Screening of ticks was performed by PCR amplification of partial 16S rRNA genes, and the 5S-23S intergenic spacer region was used for the detection of *B. burgdorferi* s.l. genospecies. The presence of *Borrelia* species was detected by amplification of 16S rRNA gene as described [16] under the following conditions: 35 cycles, 94°C-10 sec, 60°C-1 min, 72°C-90 sec. Nested PCR was performed with primer pair 16S-Bor-S4F and 16S-Bor-S3R [17] and cycling conditions included 35 cycles of initial denaturation at 94°C for10 sec, annealing at 65°C-1 min, and elongation at 72°C-90 sec.

A *B. burgdorferi* s.l. group-specific 5S-23S intergenic spacer (IGS) region PCR was used for amplification and sequencing the 16S-positive samples as described earlier [18,19], with some modifications. Cycling conditions for the first round PCR included an initial denaturation step for 1 min at 94°C, followed by 35 cycles for 1 min at 94°C, 1 min at 58°C and 2 min at 72°C, and a final extension for 15 min at 72°C. In the nested PCR, annealing temperature was decreased to 52°C and amplification was performed for 30 cycles.

The amplified products were visualized by electrophoresis in a 1% agarose gel, stained with ethidium bromide. Negative and positive control samples were included in each step of PCR amplification. Sterile deionised water was used as negative control. Samples of *B.burgdorferi* s.l. genospecies DNA obtained from culture (*B. afzelii* strain NE632, *B. garinii* strain NE11, *B. burgdorferi* s.s. strain B31, *B. valaisiana* strain VS116 and *B. lusitaniae* strain PotiB1; kindly provided by Lise Gern) as well as from ticks, were used as positive controls. To minimize contamination risks, all steps were performed in four separate rooms using sterile techniques. The sample DNA addition step to the PCR mix was performed under laminar flow.

DNA sequencing and phylogenetic analysis

Positive samples were sequenced with inner primers for 5S-23S IGS and 16S rRNA PCR, NC3 and NC4, and 16S-Bor-S4F and 16S-Bor-S3R, respectively. All PCR products were sent to the Estonian Biocenter (Tartu, Estonia) where sequencing was performed. Retrieved sequences were assembled, edited and analyzed with BioEdit v. 7.0.9.0 [20]. The UPGMA method was used for phylogenetic tree reconstruction using MEGA 5.0 package programs [21] with bootstrap analysis of 1000 replicates. The Maximum Likelihood Composite method was used for estimation of evolutionary distances in the units of base substitutions per site. Gamma distribution (shape parameter = 1) was used for modeling the rate variation among sites. The analysis involved 79 nucleotide sequences of 260 total positions in the final dataset.

Statistical analysis

The 95% confidence interval (CI) of a proportion was estimated without a correction for continuity [22,23]. Statistical significance (P) of the *B. burgdorferi* s.l. prevalence

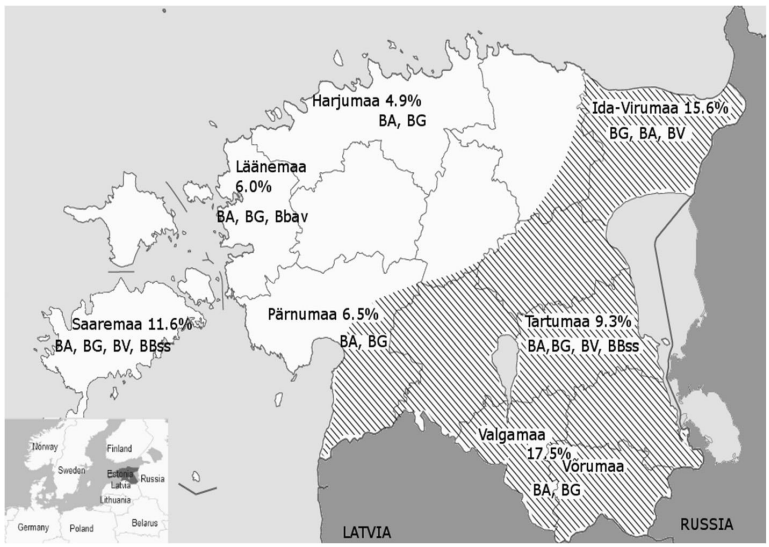

Figure 1 Tick sampling sites and *B. burgdorferi* s.l. genospecies in Estonia. Sites are grouped into regions according to their geographical and administrative locations. Regions are named according to their administrative location. The sympatric area for *I. persulcatus* and *I. ricinus* tick species is dashed according to Golovljova, I. unpublished data. BA- *B. afzelii*, BG- *B. garinii*, BV- *B. valaisiana*, Bbss- *B. burgdorferi* s.s., Bbav – *B. bavariensis*. The prevalence values of the given region are calculated according to the total tick population of the region analyzed for the presence of *B.* burgdorferi s.l.

among independent values such as tick species, stages and sites was calculated using Fisher's exact test 2 × 2 contingency table [24] and Poisson probability test.

Results

Detection of *B. burgdorferi* DNA

A total of 2833 unfed ticks, belonging to species *I. ricinus* (80.9%; 2293/ 2833) or *I. persulcatus* (19.1%; 540/ 2833), were collected from the vegetation. *I. ricinus* ticks were collected in all regions of Estonia, while *I. persulcatus* ticks were found only in Eastern and Southern Estonia (Figure 1).

B. burgdorferi s.l. DNA was found in 275 out of 2833 ticks (9.7%) collected in all 7 regions of study. The highest overall prevalence of *B. burgdorferi* s.l. (15.6%- 17.7%) was detected in the South-Eastern region of Estonia (Võrumaa/Valgamaa, Ida-Virumaa), areas sympatric for *I. ricinus* and *I. persulcatus*, as well as in the allopatric area of the Saaremaa island (10.7%) where only *I. ricinus* is found (Table 1).

The prevalence of *B. burgdorferi* s.l. in *I. persulcatus* ticks (16.3%) was significantly higher than that in *I. ricinus* (8.2%) (Table 1). The estimated infection rates within the sympatric area were twice as high in *I. persulcatus* ticks as in *I. ricinus*: 16.3% and 7.4%, respectively. However, the prevalence of *B. burgdorferi* s.l. in *I. ricinus* ticks from the sympatric areas (7.4%) did not differ much from that in allopatric areas (8.6%) (Table 1).

In mainland Estonia, the total prevalence rate of *B. burgdorferi* s.l. in both tick species in the sympatric area was significantly higher when compared to the prevalence rates in *I. ricinus* ticks in the allopatric area, 10.8% and 5.3%, respectively ($P < 0.0001$), while on Saaremaa Island the infection rate of *I. ricinus* was almost twice as high as in its mainland allopatric area (10.7% and 5.3%, respectively).

Adult ticks displayed higher infection prevalence than nymphs for both *I. ricinus* (9.4% for adults and 6.6% for nymphs) and *I. persulcatus* species (18.5% for adults and 8.0% for nymphs) (Table 1).

Detection of *B.burgdorferi* s.l. genospecies

All ticks were investigated individually for the presence of *B. burgdorferi* s.l. by PCR amplification of partial 16S rRNA and 5S-23S IGS region genes. A total of 238 tick samples out of 275 positive for *B. burgdorferi* s.l. were genotyped by sequencing of PCR products and 37 samples (13.5%) contained a mix of different *B. burgdorferi* genospecies that could not be individually identified.

B. afzelii was the most prevalent genospecies in all study regions comprising 53.5% of all *B. burgdorferi* s.l. infected ticks, and was detected in both *I. ricinus* (56.1%) and *I. persulcatus* (47.7%) ticks (Table 2). Analysis of nucleotide sequences showed that the investigated samples belong to two genomic subgroups of *B. afzelii*, VS461 and NT28 [25-27], that were detected in 62.6% and 28.6% of all

Table 1 *B. burgdorferi* s.l. prevalence in questing ticks, collected in 7 regions in Estonia

	I. ricinus						*I. persulcatus*						Prevalence, % (total No. ticks tested)	95% CI
% prevalence (No. nymphs tested)		95% CI	Prevalence, % (No. adults tested)	95% CI	Prevalence, % (total No. ticks tested)	95% CI	Prevalence,% (No. nymphs tested)	95% CI	Prevalence,% (No. adults tested)	95% CI	Prevalence, % (total No. ticks tested)	95% CI		
Ida-Virumaa	9.5 (21)	2.6-28.9	4.0 (50)	1.1-13.5	5.6 (71)	2.2-13.6	10.0 (10)	1.8-40.4	23.2 (99)	16.0-32.5	22.0 (109)	15.3-30.1	15.6 (180)	11.0-21.6
Tartumaa	3.0 (67)	0.8-10.2	10.9 (230)	7.5-15.6	9.1 (297)	6.3-12.9	7.8 (102)	4.0-14.7	10.4 (192)	6.9-15.6	9.5 (294)	6.7-13.4	9.3 (591)	7.2-11.9
Võrumaa-Valgamaa	-		9.9 (121)	5.8-16.5	9.9 (121)	5.8-16.5	-		24.6 (130)	18.0-32.7	24.6 (130)	18.0-32.7	17.5 (251)	13.3-22.7
Pärnumaa	3.5 (198)	1.7-7.1	7.8 (193)	4.8-12.4	5.6 (391)	3.8-8.4	0/1 [a]		4/6 [a]		4/7 [a]		6.5 (398)	4.5-9.4
Total in sympatric area	***3.8 (286)***	***2.2-6.8***	***9.1 (594)***	***7.0-11.7***	***7.4 (880) ¥***	***5.8-9.3***	***7.9 (113)***	***4.2-14.4***	***18.5 (79/427)***	***15.1-22.5***	***88/540 (16.3)¥***	***13.4-19.6***	***10.8 (1420)****	***9.3-12.5***
Harjumaa	3.4 (178)	1.6-7.2	6.6 (166)	3.7-11.5	4.9 (344)	3.1-7.8	-		-		-		4.9 (344)	3.1-7.8
Läänemaa	8.0 (100)	4.1-15.0	4.0 (100)	1.6-9.8	6.0 (200)	3.5-10.2	-		-		-		6.0 (200)	3.5-10.2
Total in allopatric area, mainland	***5.0 (278)***	***3.0-8.3***	***5.6 (266)***	***5.6-14.4***	***5.3 (544) ‡‡***	***3.7-7.5***							***5.3 (544)****	***3.7-7.5***
Saaremaa	9.4 (425)	7.0-12.6	11.9 (444)	9.3-15.3	10.7 (869) ‡‡	8.8-12.9	-		-		-		10.7 (869)	8.8-12.9
Total in allopatric area	***7.7 (703)***	***5.9-9.9***	***9.6 (710)***	***7.6-12.0***	***8.6 (1413)***	***7.3-10.2***							***8.6 (1413)***	***7.3-10.2***
Total	6.6 (989) ¥¥	5.2-8.3	9.4 (1304) ¥¥,‡	7.9-11.1	8.2 (2293) *	7.1-9.4	8.0 (113) ††	4.2-14.4	18.5 (427) ††,‡	15.1-22.5	16.3 (540) *	13.4-19.6	9.7 (2833)	8.7-10.9

¥,*, ‡ , **, ‡‡ Fisher's exact and Poisson probability tests P < 0.0001.
CI-Confidence Interval of a Proportion.
a - no. of infected/ tested;% prevalence as well as 95% CI were omitted due to small number of samples.

Table 2 *B. burgdorferi* s.l. genotypes in *I. ricinus* and *I. persulcatus* tick species

	I. ricinus	*I. persulcatus*	TOTAL
	No. of ticks positive/total positive (prevalence, %)	No. of ticks positive/total positive (prevalence, %)	No. of ticks positive/total positive (prevalence, %)
B. afzelii VS461	60/187 (32.1%)	32/88 (36.4%)	92/275 (33.5%)
B. afzelii NT28	37/187 (18.8%)	5/88 (5.7%)	42/275 (15.3%)
VS461+ NT28	8/187 (4.3%)	5/88 (5.7%)	13/275 (4.7%)
Total *B. afzelii*	105/ 187 (56.1%)	42/ 88 (47.7%)	147/ 275 (53.5%)
B. garinii 20047	38/187 (20.3%)	9/88 (10.2%)	47/275 (17.1%)
B. garinii NT29	0/187	25/88 (28.4%)	25/275 (9.1%)
Total *B. garinii*	38/ 187(20.3%)	34/ 88 (38.6%)	72/ 275 (26.2%)
B. valaisiana	13/ 187 (6.9%)	2/ 88 (2.3%)	15/ 275 (5.5%)
B. burgdorferi s.s.	3/ 187 (1.6%)	0/ 88	3/ 275 (1.1%)
B. bavariensis	1/ 187 (0.5%)	0/ 88	1/275 (0.4%)
Mix of several genotypes	27/ 187 (14.4%)	10/ 88 (11.4%)	37/275 (13.5%)

B. afzelii positive ticks, respectively. The nucleotide sequences of 5S-23S IGS share a high rate of similarity (99.1%-100% for VS461 and 98.3%-100% for NT28) with other *B. afzelii* sequences, reported from Russia, Belarus, Sweden, Switzerland, Italy, Turkey, Korea, China, Taiwan and Japan (Figure 2). The phylogenetic analysis of 5S-23S IGS rRNA gene revealed three lineages within *B. afzelii* subgroup VS461 and five in subgroup NT28, albeit with a bootstrap support of less than 70%. The nucleotide sequence similarity between VS461 and NT28 subgroups ranged from 96.7% to 97.9%. In addition, 8.8% of tick samples contained a mix of *B. afzelii* VS461 and NT28 (Table 3).

B. garinii was found in both tick species in all study regions. It was detected in 20.3% of *I. ricinus* and in 38.6% of *I. persulcatus* ticks, giving a total infection frequency of 26.2% of all *B. burgdorferi* s.l. infected ticks (Table 2). Estonian sequences found in the present study belong to 20047 (Eurasian) and NT29 (Asian) genomic subgroups. *B. garinii* group 20047 was the most prevalent (65.3%) and was detected in both *I. ricinu*s and *I. persulcatus* at 52.8% and 12.5% of *B. garinii* positive ticks, respectively (Table 3). *B. garinii* subgroup NT29 DNA was found only in *I. persulcatus* at 34.7% of *B. garinii* positive ticks. Estonian sequences of the 5S-23S IGS of the subgroup 20047 shared similarity rates of 96.8% -100%, with sequences derived from GenBank and detected in *Ixodes* ticks and also in rodents in Russia, Czech Republic, Turkey, Ukraine, Belarus, Italy, UK, Italy, France, Switzerland and China. Within subgroup NT29, the Estonian sequences were identical with those amplified from ticks, rodents and human samples from Russia, Latvia and China with similarity rates from 98.9% to 100%. Phylogenetic analysis based on 5S-23S IGS showed that Estonian samples reported in the current study are distributed within seven lineages in subgroup 20047 and five lineages in subgroup NT29 albeit with low bootstrap values (Figure 2).

B. valaisiana DNA was detected in *I. ricinus* ticks collected on Saaremaa Island and in both *I. ricinus* and *I. persulcatus* ticks collected in Tartumaa and Ida-Virumaa in the sympatric mainland area with an overall infection rate of 5.5% (Table 2). The phylogenetic analysis based on 5S-23S IGS showed that Estonian sequences belong to *B. valaisiana* group VS116 and cluster with *B. valaisiana* sequences reported from Netherlands, Czech Republic and UK with similarity rates from 98.8% to 100% (Figure 2).

B. burgdorferi s.s. was found in three *I. ricinus* ticks (1.1%) collected from Tartumaa and Saaremaa Island. Analysis of *B. burgdorferi* s.s. samples showed that all three Estonian sequences are identical to each other and cluster with sequences from *Ixodes* ticks and rodents from USA and Europe as well as with sequences from patients from the Czech Republic.

B. bavariensis DNA was detected in one *I. ricinus* tick (0.4%) collected in Läänemaa. The nucleotide sequence of 5S-23S IGS of the Estonian sample was identical to the sequence of *B. garinii* strain PBi from Germany, which is a prototype strain for *B. bavariensis*.

Discussion

Estonia is situated in the unique area where the ranges of *I. ricinus* and *I. persulcatus* overlap in the Eastern and Southern parts. This fact may play an important role in the distribution and diversity of tick-borne pathogens. The study describes the first survey regarding the presence, the prevalence and genetic characteristics of *B. burgdorferi* s.l. genospecies in questing ticks collected from different sites in *I. ricinus* allopatric areas as well as in the areas sympatric for both tick species in Estonia.

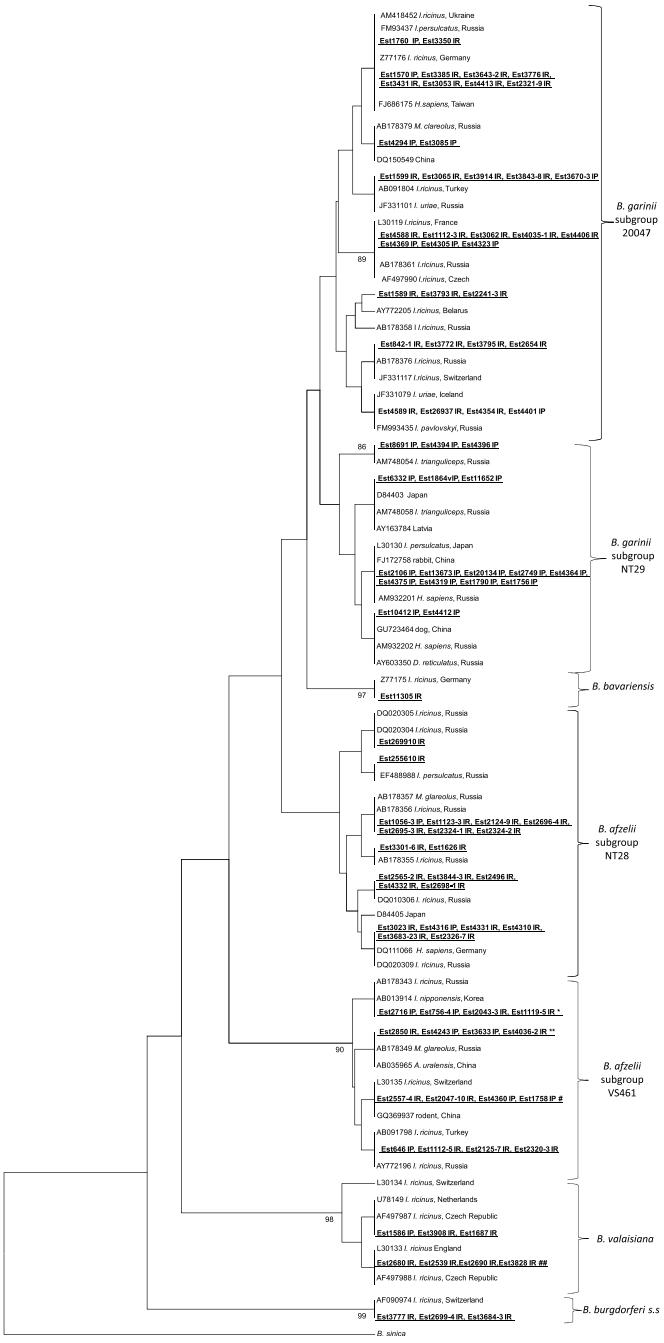

Figure 2 (See legend on next page.)

(See figure on previous page.)
Figure 2 Phylogenetic tree (UPGMA) based on the partial sequences of *B. burgdorferi*. s.l. 5S-23S rRNA IGS (235–253 bp). A sequence of *B. sinica* retrieved from GenBank was included as the outrgroup. Only support values exceeding 70% are shown. Sequences detected in the present study are shown in bold and underlined, and followed by label of tick species from which *B. burgdorferi* s.l. was amplified (IR- *I. ricinus*, IP- *I. persulcatus*). *identical to Est2043-2 IR, Est2559-3 IR, Est4268 IR, Est4583 IR, Est4396 IP, Est4397 IR, Est4400 IR, Est4374 IP, Est4372 IP, Est4373 IP, Est4361 IP, Est1116-2 IR, Est2557-7 IR, Est3915 IR, Est1642 IR, Est3703-4 IP, Est3670-1 IP, Est2325-2 IR, Est1625 IR. **identical to Est4255 IP, Est633-1 IP, Est1056-2 IP, Est1635 IR, Est4317 IP, Est4301 IP, Est2254-3 IR, Est3683-20 IR, Est1644 IR, Est1165-1 IP Est771 IP, Est1828 IP, Est4036-3 IR, Est2895-7 IR, Est3697-1 IP, Est3699-3 IP, Est2848 IR, Est2700-3 IR, Est2698-5 IR, Est2696-2 IR, Est2696-1 IR, Est2694-5 IR, Est1774 IP. # identical to Est3918 IR, Est4586 IR, Est2112 IR, Est1639 IR, Est4359 IP, Est2320-7 IR, Est2319-3 IR, Est2253-1 IR, Est2244-10 IR, Est2692-2 IR, Est2304 IR, Est2031 IR, Est1758 IP. ##identical to Est3829 IR,Est3833 IR, Est3925 IR, Est 2242–9 IR, Est2696-8 IR, Est2699-9 IR, Est3840-4 IR.

The overall infection rate of ticks collected from vegetation (n = 2283) was 9.7% as detected by PCR. Similar results have been reported from Sweden (11%) [28], Lithuania (10.2%) [29], Belarus (9.4%) [30] and the Moscow region in Russia (13.3%) [31]. In contrast, in neighboring Latvia, which also constitutes a sympatric area of *I. ricinus* and *I. persulcatus*, the *B. burgdorferi* s.l. prevalence in questing ticks was as high as 25.3% [32]. In neighboring Finland the prevalence of LB spirochetes in ticks is lower (5.1%) [15], and similar to the infection rates detected in the current study on the mainland where only *I. ricinus* is distributed. However, the comparison of prevalence in the neighboring countries mentioned above is difficult due to the differences in sensitivity for the different methods used for *Borrelia* detection.

Both *I. ricinus* and *I. persulcatus* tick species are important vectors of *B. burgdorferi* s.l. in Eurasia. Moreover, it has been suggested that *I. persulcatus* ticks are more efficient vectors than *I. ricinus* for *B. burgdorferi* s.l. spirochetes in the natural foci [33]. This notion is in correspondence with the results of the current study and our previous investigations of *B. miyamotoi* [34] and tick-borne encephalitis virus (TBEV) [35], which showed significantly higher prevalences of these tick-borne pathogens (TBPs) in *I. persulcatus* than in *I. ricinus*. In addition, the reported infection rates of these TBPs from sympatric areas are higher than those from *I. ricinus* ranges. We suggest that in the Eastern Estonia where the *I. ricinus* and *I. persulcatus* ranges overlap, certain more favorable environmental, biotic or abiotic factors, may play a role in the enhanced of circulation of TBPs.

Table 3 *B. afzelii* and *B. garinii* genospecies in *I. ricinus* and *I. persulcatus* ticks

	I. ricinus	*I.persulcatus*	Total
	B. afzelii (No.147)		
VS461	60/147 (40.8%)*	32/147 (21.8%)*	92/147 (62.6%)
NT28	37/147 (25.2%)**	5/147 (3.4%)**	42/147 (28.6%)
VS461/NT28 mix	8/147 (5.4%)	5/147 (3.4%)	13/147 (8.8%)
	B. garinii (No. 72)		
20047	38/72 (52.8%)†	9/72 (12.5%)†	47/72 (65.3%)
NT29	0/72	25/72 (34.7%)	25/72 (34.7%)

*, **, † Fisher's exact test P < 0.001.

According to the model, based on temperature, climate and vegetation data, recently presented by Estrada-Peña *et al.* [36], the Western coastline of Estonia has a different pattern of tick distribution compared to that of mainland Estonia. This may be one of the reasons why the prevalence of *B. burgdorferi* s.l. in ticks from Saaremaa Island, as well as previously reported data from Vormsi Island [15], differs from the infection rates in ticks from mainland Estonia. Similar findings have also been shown for *B. miyamotoi* [34]. The high prevalence rates of *B. burgdorferi* s.l. in ticks from Saaremaa Island are in correspondence with data on the incidence rate of Lyme borreliosis, as the annual reported numbers of LB cases in Saaremaa are the highest in Estonia during the last decade. However, there might be other factors (abundance of small and large mammals, environment, microclimate etc.) that make the island a unique area with more favorable conditions for pathogen circulation.

Although adult ticks are not considered significant in the infection cycle of *B. burgdorferi* s.l., infection rates in adults are higher than in nymphs, because adult ticks have fed twice on different hosts [37]. In the current study the infection rates of adult ticks vs nymphs are higher for both *I. ricinus* and *I. persulcatus*, and are in correspondence with the values reported from regions with low infection rates in Europe in the meta analysis by Rauter and Hartung [37], as well as to those from the neighboring countries Finland [15], Latvia [38], Sweden [28].

B.burgdorferi s.l. genospecies

The presence of *B. afzelii* and *B. garinii* in Estonian ticks has been reported previously [15,39]. As in most European countries, the most prevalent *B. burgdorferi* s.l. genospecies in Estonian ticks were *B. afzelii* (53.5%) and *B. garinii* (26.2%) with prevalence rates in correspondence with data from neighboring regions Russia [40], Finland [41] and Latvia [38]. Several studies from Europe indicate a wide spread of B. *afzelii* and its subgroups NT28 and VS461 [19,25] in different regions of Europe and Asia as well as in different vector species [27,42]. The fact that both genetic groups of *B. afzelii* and even a mix of these subgroups were found in *I. ricinus* as well as in *I. persulcatus* ticks in Estonia indicates a co-circulation of these genetic subgroups and their variants in the same natural foci and a

sharing of vectors and hosts with no specific limitations. The genetic variants revealed in this study within both NT28 and VS461 subgroups also support the notion of a genetic heterogeneity of the *B. afzelii* genospecies circulating in Europe [27,43].

B. garinii is mostly associated with avian reservoirs, especially migratory passerines that can carry infected ticks over distances and even between continents [8]. Two genetic subgroups of *B. garinii* have been reported: subgroup 20047 ("European") that circulates in *I. ricinus* and *I. persulcatus* and has a wide geographical distribution over Eurasia, and subgroup NT29 ("Asian") [19], which has never been reported from *I. ricinus* up to date, and thus has a more limited geographic distribution. Thus, the detection of *B. garinii* subgroup NT29 in *I. persulcatus* ticks in Eastern Estonia makes this region, as well as areas of Eastern Latvia [32], a unique region in Eastern Europe of *B. garinii* subgroup NT29 circulation due to distribution of its main vector, *I. persulcatus*. The high nucleotide sequence identity of Estonian samples to the sequences reported from European and Asian countries and the detection of at least 11 genetic variants of *B. garinii* within both subgroups 20047 and NT29 are in agreement with the proposed wide geographical distribution of *B. garinii* as well as genetic heterogeneity of this *Borrelia* species [44,45].

B. valaisiana is also associated mostly with avian reservoirs [46,47], and our previous studies reported the detection of this *Borrelia* genospecies as well as *B. garinii* in ticks removed from migratory passerines [48]. In the current study we report the detection of *B. valaisiana* in questing ticks with infection rates similar to those reported from Sweden (6%) [28] and Norway (6%) [49]. To date there have only been rare reports on the detection of *B. valaisiana* in *I. persulcatus* ticks [50,51]. However, the detection of *B. valaisiana* in *I. persulcatus* ticks presented in the current study, as well as in Latvia [52] and in the Baltic regions of Russia [53], suggests that in areas sympatric for both *I. ricinus* and *I. persulcatus*, *B. valaisiana* may exchange tick vectors. Our studies on TBEV [35] and *B. miyamotoi* [34] also revealed that under conditions of *I. ricinus* and *I. persulcatus* sympatry, these TBPs may switch to a different tick vector and utilize both tick species as vectors. The events of sharing between tick species may also lead to adaptation of TBPs to a new vector, resulting in the spread of TBPs to new areas.

In European countries, *B. burgdorferi* s.s. is prevalent in *I. ricinus* ticks at different rates. In this study we report the presence of this *Borrelia* species in the Estonian *I. ricinus* population for the first time, albeit at a low rate (0.1%). Although overall data presented previously in a meta analysis have shown about 16% of ticks to be infected with *B. burgdorferi* s.s. in Norway, Finland, Sweden and Estonia [37], data from the Baltic regions of Russia [53], Sweden [28], Latvia [38], Finland [15], and Belarus [30] revealed infection rates ranging from 0.3 to 2.1%, which correlates with the results of the current study.

In the present study we also report for the first time the presence of *B. bavariensis* in ticks in Estonia. This species seems to be limited to Central Europe [54] and has been reported from Switzerland [55], Austria, Germany and the Czech Republic [56]. While a high prevalence of *B. bavariensis*-like *Borrelia* in *I. persulcatus* ticks has been reported in Mongolia [57], this *Borrelia* genospecies was detected only in *I. ricinus* in our study.

A part of the ticks collected in the regions of this study and investigated for the presence of *B. burgdorferi* s.l., were also analyzed previously for *B. miyamotoi*, *A. phagocytophilum* and TBEV [34,35,58]. However, no co-infections with TBEV (628/2833) [35] or *A. phagocytophilum* (739/2833) [58] were found. Double infection with *B. miyamotoi* and *B. burgdorferi* s.l. was shown only for 4 ticks out of 2458, which were analyzed for both pathogens, as recently described by Geller *et al.* [34].

Conclusions

The recent study showed the circulation of five genospecies of *B. burgdorferi* s.l. complex, at least four of which, *B. afzelii*, *B. garinii*, *B. bavariensis* and *B. burgdorferi* s.s., are of medical importance. This study, as well as our previous investigations [34,35], presented an exchange of TBPs between the natural tick vectors and sympatric tick species, that may indicate adaptation to a new vector species and lead to the expansion of TBPs to new geographical ranges. As the incidence rate of LB in Estonia is the highest in the Baltic regions and the annual number of LB cases remains high, the monitoring of *Borrelia* in its natural foci as well as in its natural hosts and tick vectors is of public health importance. This may also help the understanding of the ecology of TBPs and their vectors, as well as environmental, biotic and abiotic factors that may influence the abundance of ticks, prevalence of TBPs and the morbidity rates of tick-borne diseases.

Abbreviations

LB: Lyme borreliosis; PCR: Polymerase chain reaction; rRNA: Ribosomal RNA; IGS: Internal transcribed spacer; CI: Confidence interval; TBEV: Tick-borne encephalitis virus; TBP(s): Tick-borne pathogen(s).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JG, OK and IG carried out the field collections and morphological identifications. JG, LN, OK and IG carried out diagnostic protocols. JG, LN and IG carried out genetic studies, sequence alignments and drafted the manuscript. JG and IG participated in the design of the study and performed the statistical analysis. JG, LN, OK and IG conceived the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Preferences

1. Hubalek Z: **Epidemiology of Lyme borreliosis.** *Curr Probl Dermatol* 2009, **37:**31–50.
2. Health Board: *Estonian communicable disease bulletins.* http://www.terviseamet.ee/en/cdc/estonian-communicable-disease-bulletins-2005-2012.html.
3. WHO: *Centralized information system for infectious diseases.* http://data.euro.who.int/cisid/.
4. Rudenko N, Golovchenko M, Grubhoffer L, Oliver JH Jr: **Updates on *Borrelia burgdorferi* sensu lato complex with respect to public health.** *Ticks Tick Borne Dis* 2011, **2:**123–128.
5. Humair P, Gern L: **The wild hidden face of Lyme borreliosis in Europe.** *Microbes Infect* 2000, **2:**915–922.
6. Hanincova K, Schafer SM, Etti S, Sewell HS, Taragelova V, Ziak D, Labuda M, Kurtenbach K: **Association of *Borrelia afzelii* with rodents in Europe.** *Parasitology* 2003, **126:**11–20.
7. Huegli D, Hu CM, Humair PF, Wilske B, Gern L: ***Apodemus* species mice are reservoir hosts of *Borrelia garinii* OspA serotype 4 in Switzerland.** *J Clin Microbiol* 2002, **40:**4735–4737.
8. Comstedt P, Bergstrom S, Olsen B, Garpmo U, Marjavaara L, Mejlon H, Barbour AG, Bunikis J: **Migratory passerine birds as reservoirs of Lyme borreliosis in Europe.** *Emerg Infect Dis* 2006, **12:**1087–1095.
9. Hanincova K, Taragelova V, Koci J, Schafer SM, Hails R, Ullmann AJ, Piesman J, Labuda M, Kurtenbach K: **Association of *Borrelia garinii* and *B. valaisiana* with songbirds in Slovakia.** *Appl Environ Microbiol* 2003, **69:**2825–2830.
10. Baranton G, Postic D, Grimont PA, Assous M, Piffaretti JC, Boerlin P, Saint Girons I: **Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis.** *Int J Syst Bacteriol* 1992, **42:**378–383.
11. Talleklint L, Jaenson TG: **Transmission of *Borrelia burgdorferi* s.l. from mammal reservoirs to the primary vector of Lyme borreliosis, *Ixodes ricinus* (Acari: Ixodidae), in Sweden.** *J Med Entomol* 1994, **31:**880–886.
12. Richter D, Debski A, Hubalek Z, Matuschka FR: **Absence of Lyme disease spirochetes in larval *Ixodes ricinus* ticks.** *Vector Borne Zoonotic Dis* 2012, **12:**21–27.
13. Gern L, Rais O: **Efficient transmission of *Borrelia burgdorferi* between cofeeding *Ixodes ricinus* ticks (Acari: Ixodidae).** *J Med Entomol* 1996, **33:**189–192.
14. Randolph SE, Gern L, Nuttall PA: **Co-feeding ticks: Epidemiological significance for tick-borne pathogen transmission.** *Parasitol Today* 1996, **12:**472–479.
15. Makinen J, Vuorinen I, Oksi J, Peltomaa M, He Q, Marjamaki M, Viljanen MK: **Prevalence of granulocytic *Ehrlichia* and *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks collected from Southwestern Finland and from Vormsi Island in Estonia.** *APMIS* 2003, **111:**355–362.
16. Fukunaga M, Takahashi Y, Tsuruta Y, Matsushita O, Ralph D, McClelland M, Nakao M: **Genetic and phenotypic analysis of *Borrelia miyamotoi* sp. nov., isolated from the ixodid tick *Ixodes persulcatus*, the vector for Lyme disease in Japan.** *Int J Syst Bacteriol* 1995, **45:**804–810.
17. Fomenko NV, Livanova NN, Borgoiakov V, Kozlova IV, Shulaikina IV, Pukhovskaia NM, Tokarevich KN, Livanov SG, Doroshchenko EK, Ivanov LI: **Detection of *Borrelia miyamotoi* in ticks *Ixodes persulcatus* from Russia.** *Parazitologiia* 2010, **44:**201–211.
18. Rar VA, Fomenko NV, Dobrotvorsky AK, Livanova NN, Rudakova SA, Fedorov EG, Astanin VB, Morozova OV: **Tickborne pathogen detection, Western Siberia, Russia.** *Emerg Infect Dis* 2005, **11:**1708–1715.
19. Postic D, Assous MV, Grimont PA, Baranton G: **Diversity of *Borrelia burgdorferi* sensu lato evidenced by restriction fragment length polymorphism of rrf (5S)-rrl (23S) intergenic spacer amplicons.** *Int J Syst Bacteriol* 1994, **44:**743–752.
20. Hall T: **BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT.** *Nucleic Acids Symp Ser* 1999, **41:**95–98.
21. Tamura K DJ, Nei M, Kumar S: **MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0.** *Mol Biol Evol* 2007, **24:**1596–1599.
22. Newcombe RG: **Two-sided confidence intervals for the single proportion: comparison of seven methods.** *Stat Med* 1998, **17:**857–872.
23. Wilson EB: **Probable Inference, the Law of Succession, and Statistical Inference.** *J Am Stat Assoc* 1927, **22:**209–212.
24. Fisher RA: **On the interpretation of χ2 from contingency tables, and the calculation of P.** *J R Stat Soc* 1922, **85:**87–94.
25. Masuzawa T, Komikado T, Iwaki A, Suzuki H, Kaneda K, Yanagihara Y: **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* 1996, **142:**77–83.
26. Picken RN, Cheng Y, Strle F, Picken MM: **Patient isolates of *Borrelia burgdorferi* sensu lato with genotypic and phenotypic similarities of strain 25015.** *J Infect Dis* 1996, **174:**1112–1115.
27. Fadeeva IA, Nefedova VV, Korenberg EI, Gorelova NB: **Genetic variants of *Borrelia afzelii*, a pathogen of the ixodes tick borrelioses.** *Mol Gen Mikrobiol Virusol* 2005, **3:**18–22.
28. Fraenkel CJ, Garpmo U, Berglund J: **Determination of novel *Borrelia* genospecies in Swedish *Ixodes ricinus* ticks.** *J Clin Microbiol* 2002, **40:**3308–3312.
29. Motiejunas L, Bunikis J, Barbour AG, Sadziene A: **Lyme borreliosis in Lithuania.** *Scand J Infect Dis* 1994, **26:**149–155.
30. Reye AL, Stegniy V, Mishaeva NP, Velhin S, Hubschen JM, Ignatyev G, Muller CP: **Prevalence of tick-borne pathogens in *Ixodes ricinus* and *Dermacentor reticulatus* ticks from different geographical locations in Belarus.** *PLoS One* 2013, **8:**e54476.
31. Masuzawa T, Kharitonenkov IG, Kadosaka T, Hashimoto N, Kudeken M, Takada N, Kaneda K, Imai Y: **Characterization of Borrelia burgdorferi sensu lato isolated in Moscow province–a sympatric region for Ixodes ricinus and Ixodes persulcatus.** *Int J Med Microbiol* 2005, **294:**455–464.
32. Ranka R, Bormane A, Salmina K, Baumanis V: **Identification of three clinically relevant *Borrelia burgdorferi* sensu lato genospecies by PCR-restriction fragment length polymorphism analysis of 16S-23S ribosomal DNA spacer amplicons.** *J Clin Microbiol* 2004, **42:**1444–1449.
33. Korenberg EI, Kovalevskii YV, Levin ML, Shchyogoleva TV: **The prevalence of *Borrelia burgdorferi* sensu lato *in Ixodes persulcatus* and *I. ricinus* ticks in the zone of their sympatry.** *Folia Parasitol (Praha)* 2001, **48:**63–68.
34. Geller J, Nazarova L, Katargina O, Jarvekulg L, Fomenko N, Golovljova I: **Detection and genetic characterization of relapsing fever spirochete *Borrelia miyamotoi* in Estonian Ticks.** *PLoS One* 2012, **7:**e51914.
35. Katargina O, Russakova S, Geller J, Kondrusik M, Zajkowska J, Zygutiene M, Bormane A, Trofimova J, Golovljova I: **Detection and characterization of tick-borne encephalitis virus in Baltic countries and eastern Poland.** *PLoS One* 2013, **8:**e61374.
36. Estrada-Pena A, Farkas R, Jaenson TG, Koenen F, Madder M, Pascucci I, Salman M, Tarres-Call J, Jongejan F: **Association of environmental traits with the geographic ranges of ticks (Acari: Ixodidae) of medical and veterinary importance in the western Palearctic. A digital data set.** *Exp Appl Acarol* 2013, **59:**351–366.
37. Rauter C, Hartung T: **Prevalence of *Borrelia burgdorferi* sensu lato genospecies in *Ixodes ricinus* ticks in Europe: a metaanalysis.** *Appl Environ Microbiol* 2005, **71:**7203–7216.
38. Etti S, Hails R, Schafer SM, De Michelis S, Sewell HS, Bormane A, Donaghy M, Kurtenbach K: **Habitat-specific diversity of *Borrelia burgdorferi* sensu lato in Europe, exemplified by data from Latvia.** *Appl Environ Microbiol* 2003, **69:**3008–3010.
39. Postic D, Korenberg E, Gorelova N, Kovalevski YV, Bellenger E, Baranton G: ***Borrelia burgdorferi* sensu lato in Russia and neighbouring countries: high incidence of mixed isolates.** *Res Microbiol* 1997, **148:**691–702.
40. Korotkov Iu S, Kislenko GS, Burenkova LA, Rudnikova NA, Karan LS: **Spatial and temporal variability of *Ixodes ricinus* and *Ixodes persulcatus* infection with the Lyme disease agent in Moscow Region.** *Parazitologiia* 2008, **42:**441–451.
41. Junttila J, Peltomaa M, Soini H, Marjamaki M, Viljanen MK: **Prevalence of *Borrelia burgdorferi* in *Ixodes ricinus* ticks in urban recreational areas of Helsinki.** *J Clin Microbiol* 1999, **37:**1361–1365.
42. Fadeeva IA, Korenberg EI, Nefedova VV, Andreichuk Iu V, Markov AV, Shaginian IA: **Genetic heterogeneity of *Borrelia afzelii* in the natural focus of the Middle Urals.** *Zh Mikrobiol Epidemiol Immunobiol* 2006, **3:**27–30.
43. Bunikis J, Garpmo U, Tsao J, Berglund J, Fish D, Barbour AG: **Sequence typing reveals extensive strain diversity of the Lyme borreliosis agents *Borrelia burgdorferi* in North America and *Borrelia afzelii* in Europe.** *Microbiology* 2004, **150:**1741–1755.
44. Comstedt P, Asokliene L, Eliasson I, Olsen B, Wallensten A, Bunikis J, Bergstrom S: **Complex population structure of Lyme borreliosis**

group spirochete *Borrelia garinii* in subarctic Eurasia. *PLoS One* 2009, **4**:e5841.
45. Nefedova VV, Korenberg EI, Gorelova NB: **Genetic variants of *Borrelia garinii*, a widely spread Eurasian pathogen of ixodid tick borreliosis.** *Mol Gen Mikrobiol Virusol* 2010, **3**:7–12.
46. Hubalek Z: **An annotated checklist of pathogenic microorganisms associated with migratory birds.** *J Wildl Dis* 2004, **40**:639–659.
47. Humair PF, Postic D, Wallich R, Gern L: **An avian reservoir (*Turdus merula*) of the Lyme borreliosis spirochetes.** *Zentralbl Bakteriol* 1998, **287**:521–538.
48. Geller J, Nazarova L, Katargina O, Leivits A, Jarvekulg L, Golovljova I: **Tick-borne pathogens in ticks feeding on migratory passerines in Western part of Estonia.** *Vector Borne Zoonotic Dis* 2013, **13**:443–448.
49. Jenkins A, Hvidsten D, Matussek A, Lindgren PE, Stuen S, Kristiansen BE: ***Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks from Norway: evaluation of a PCR test targeting the chromosomal flaB gene.** *Exp Appl Acarol* 2012, **58**:431–439.
50. Saito K, Ito T, Asashima N, Ohno M, Nagai R, Fujita H, Koizumi N, Takano A, Watanabe H, Kawabata H: **Case report: *Borrelia valaisiana* infection in a Japanese man associated with traveling to foreign countries.** *Am J Trop Med Hyg* 2007, **77**:1124–1127.
51. Lesnyak O, Laikovskaya E, Kufko I, Bruinink H, Baranova N, Rijpkema S: **Clinical features of Lyme borreliosis in the middle Urals and distribution of *Borrelia burgdorferi* sensu lato species in local *Ixodes persulcatus* ticks.** *Zentralbl Bakteriol* 1998, **288**:111–119.
52. Bormane A, Lucenko I, Duks A, Mavtchoutko V, Ranka R, Salmina K, Baumanis V: **Vectors of tick-borne diseases and epidemiological situation in Latvia in 1993–2002.** *Int J Med Microbiol* 2004, **293**(Suppl 37):36–47.
53. Alekseev AN, Dubinina HV, Van De Pol I, Schouls LM: **Identification of *Ehrlichia* spp. and *Borrelia burgdorferi* in *Ixodes* ticks in the Baltic regions of Russia.** *J Clin Microbiol* 2001, **39**:2237–2242.
54. Franke J, Hildebrandt A, Dorn W: **Exploring gaps in our knowledge on Lyme borreliosis spirochaetes - Updates on complex heterogeneity, ecology, and pathogenicity.** *Ticks Tick Borne Dis* 2013, **4**:11–25.
55. Gern L, Douet V, Lopez Z, Rais O, Moran Cadenas F: **Diversity of *Borrelia* genospecies in *Ixodes ricinus* ticks in a Lyme borreliosis endemic area in Switzerland identified by using new probes for reverse line blotting.** *Ticks Tick Borne Dis* 2010, **1**:23–29.
56. Skuballa J, Petney T, Pfaffle M, Oehme R, Hartelt K, Fingerle V, Kimmig P, Taraschewski H: **Occurrence of different *Borrelia burgdorferi* sensu lato genospecies including *B. afzelii*, *B. bavariensis*, and *B. spielmanii* in hedgehogs (*Erinaceus* spp.) in Europe.** *Ticks Tick Borne Dis* 2012, **3**:8–13.
57. Scholz HC, Margos G, Derschum H, Speck S, Tserennorov D, Erdenebat N, Undraa B, Enkhtuja M, Battsetseg J, Otgonchimeg C, *et al*: **High prevalence of genetically diverse *Borrelia bavariensis*-like strains in *Ixodes persulcatus* from Selenge Aimag, Mongolia.** *Ticks Tick Borne Dis* 2013, **4**:89–92.
58. Katargina O, Geller J, Alekseev A, Dubinina H, Efremova G, Mishaeva N, Vasilenko V, Kuznetsova T, Jarvekulg L, Vene S, *et al*: **Identification of *Anaplasma phagocytophilum* in tick populations in Estonia, the European part of Russia and Belarus.** *Clin Microbiol Infect* 2012, **18**:40–46.

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