THESIS ON NATURAL AND EXACT SCIENCES B109

Tick-Borne Pathogens Circulating in Estonia (Tick-Borne Encephalitis Virus, *Anaplasma phagocytophilum*, *Babesia* species): Their Prevalence and Genetic Characterization

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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.

Olga Katargina

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Eestis tsirkuleerivate puukide abil ülekantavad patogeenid (puukentsefaliidiviirus, *Anaplasma phagocytophilum, Babesia* liigid): nende levik ja geneetiline iseloomustus

OLGA KATARGINA

TTÜ KIRJASTUS

To my family

CONTENTS

INTRODUCTION

A WHO World Health Report (23 August 2007) foresaw emerging infectious diseases as major threats to global public health in the 21st century. Approximately 60% of these emerging infectious diseases are caused by zoonotic agents, and a majority of them (72 %) originate in wildlife (Jones et al., 2008). A zoonosis is any disease or infection that is naturally transmissible between vertebrate animals and humans. Transmission between animals and/or humans occurs indirectly by vectors or directly (without vectors) through excreta, bites, food and etc. Vectors are the transmitters of disease-causing organisms that carry pathogens from one host to another. Arthropods, like ticks, mosquitoes and fleas are the most important zoonotic pathogen vectors in Europe. In our work we emphasised the study of tick-borne pathogens.

Tick-borne pathogens (TBP), transmitted by principally *Ixodes ricinus* and *Ixodes persulcatus* ticks, are widely spread and have a large impact on human morbidity. Tick-borne encephalitis virus is a well studied pathogen and the disease caused by it, tick-borne encephalitis (TBE), is widely distributed and well recognized both in Europe and in Estonia. In contrast, other TBP, i.e. the less known ones, like *Anaplasma* and *Babesia,* are not so well characterized, in spite of the fact that human granulocytic anaplasmosis (HGA) and babesiosis in humans have been reported during the last decades. The importance of TBP studies in Estonia is defined by the particularity that only in the Baltic countries (Estonia and Latvia), there is an overlap of the distribution of the two tick species *Ixodes ricinus* and *Ixodes persulcatus*. The co-existence of different tick species could serve as a unique model for investigation of TBP and the relationships of different vectors in the Baltic countries.

The most harmful TBP is tick-borne encephalitis virus (TBEV), belongs to genus *Flavivirus,* family *Flaviviridae*. TBEV causes encephalitis and/or meningitis or even paralysis in infected persons. The highest incidence rates of TBE in Europe are found in Estonia. In a previous study we demonstrated that all three subtypes of TBEV circulate in Estonia (Golovljova et al., 2004). The Western or European subtype (TBEV-Eur) is transmitted by *I. ricinus* ticks, and causes TBE with a mortality rate of about 1% in humans. The Far-Eastern subtype (TBEV-FE) was first isolated in the Far-East and is carried by *I. persulcatus.* A mortality rate of up to 25% for this subtype has been reported in Far-East Russia. The Siberian subtype (TBEV-Sib) was first isolated in Siberia and is also carried by *I. persulcatus* ticks. TBEV-Sib may cause a chronic or persistent form of human infection in Siberia, but this form of disease has never been detected in the Baltic countries, although the prevalence of this subtype in the tick population is high in Estonia and Latvia. Thus, the first aim of the current study was to analyse the genetic and phylogenetic properties of Estonian strains belonging to TBEV-Sib subtype.

Anaplasma phagocytophilum is a gram-negative intracellular bacterium transmitted by ticks, and infects mammalian granulocytes. It causes human

granulocytic anaplasmosis (HGA) - an acute, nonspecific febrile illness characterized by headache, myalgia, malaise, as well as tick-borne fever (TBF) in ruminants. The number of HGA and TBF cases in Europe is continuously increasing. Several new variants and lineages of *A. phagocytophilum* have been detected in European natural foci and they display different host/vector tropism and pathogenicity (Massung et al., 2002; Petrovec et al., 2002). Studies in different European countries reported seroprevalence rates from zero to 28% in the examined human populations (Strle, 2004). Up to date, only one case of HGA has been reported in Estonia (Prukk et al., 2003), while the presence and distribution of *A. phagocytophilum* in the Estonian tick population was generally unknown. The next aim of the current thesis was to detect *A. phagocytophilum* in the tick population and characterize it genetically by sequencing three of its genes (16S RNA, *groESL*, and *ankA*).

Babesia species are protozoan parasites, which infect mammalian erythrocytes, thereby causing lysis, which in turn results in anemia, hyperbilirubinuria, hemoglobulinuria and possibly organ failure. Babesiosis is caused by *Babesia microti* in North America and *Babesia divergens* and *Babesia* sp. EU1 in Europe. During the last decade increasing numbers of human infections have been reported in USA and Europe (Homer et al., 2000; Kjemtrup and Conrad, 2000). Around 40 human cases of babesiosis, with up to a 42% mortality rate, have been confirmed In Europe (Grey et al., 2010). In neighbouring Scandinavian and Baltic countries, cases of human babesiosis have been reported in Finland and Sweden (Uhnoo et al., 1992; Haapasalo et al., 2010), and the circulation of *Babesia* in tick populations was reported in Lithuania and Norway (Radzijevskaja et al., 2008). Up to date, no human cases due to *Babesia* spp. up to date have been reported in Estonia, and the presence and prevalence of *Babesia* spp. in Estonia remained unknown. Therefore, the last aim of this study was to investigate the presence and prevalence of *Babesia* spp. in the tick population and to genetically characterize *Babesia* spp. in Estonia.

ORIGINAL PUBLICATIONS

The present dissertation is based on the following publications:

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

2. **Katargina, O**., Geller, J., Alekseev, A., Dubinina, H., Efremova, G., Mishaeva, N., Vasilenko, V., Kuznetsova, T., Järvekülg, L., Vene, S., Lundkvist, Å. and Golovljova, I. (2011). Identification of *Anaplasma phagocytophilum* in tick populations in Estonia, the European Part of Russia and Belarus. Accepted for publication in *Clinical Microbiology and Infection*: December 23, 2010. Online Ahead of Print: January 2, 2011.

3. **Katargina, O.**, Geller, J., Vasilenko, V., Kuznetsova, T., Järvekülg, L., Vene, S., Lundkvist, Å. and Golovljova, I. (2011). Detection and characterization of *Babesia* species in *Ixodes* ticks in Estonia. Accepted for publication in *Vector Borne and Zoonotic Disease:* January 4, 2011. Online Ahead of Print: March 11, 2011.

ABBREVIATIONS

aa	amino acid
anch C	nascent C nucleocapsid protein
AnkA	gene of ankyrin
bp	base pair
C	nucleocapsid protein
cDNA	complementary DNA
DNA	deoxyribonucleic acid
dNTP	desoxyribonucleotide triphosphate
E	envelope protein
EDTA	ethylene-diamine-tetra-acetic acid
ER	endoplasmic reticulum
groESL	gene of heat-shock protein
HGA	human granulocytic anaplasmosis
M	membrane protein
MIR	minimum infection rate
NCR	noncoding region
nt	nucleotide
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
prM	precursor of M protein
RLB	reverse line blot
RNA	ribonucleic acid
RT-PCR	reverse transcriptase PCR
16S rRNA	small subunits of ribosomal RNA
18S rRNA	small subunits of ribosomal RNA
SDS	sodium dodecyl sulfate
SSPE	saline sodium phosphate-EDTA
TBD	tick-borne disease
TBE	tick-borne encephalitis
TBEV	tick-borne encephalitis virus
TBEV-Eur	tick-borne encephalitis virus - European subtype
TBEV-FE	tick-borne encephalitis virus – Far Eastern subtype
TBEV-Sib	tick-borne encephalitis virus - Siberian subtype
TBF	tick-borne fever
TBP	tick-borne pathogen

1. REVIEW OF THE LITERATURE

1.1. Tick-borne encephalitis virus

TBE virus (TBEV) belongs to the genus *Flavivirus* within the family *Flaviviridae*. The family *Flaviviridae* consists of three genera: *Pestivirus* genus that includes animal pathogens as e.g. classical swine fever virus (CSFV), bovine diarrhea virus (BVDV) and border disease virus (BDV) of sheep; *Hepacivirus* genus that includes hepatitis C virus (HCV), GB virus B (GBV-B) and the unclassified viruses DB virus A (GBV-A) and GB virus C (GBV-C); and genus *Flavivirus*.

The flaviviruses derive their name from *flavus* (yellow) which refers to the type species, yellow fever virus. The genus *Flavivirus* consists of more than 70 viruses, half of which are mosquito-borne (Aroa virus group, dengue virus group, Japanese encephalitis group, Kokobera virus group, Ntaya virus group, Spondweni virus group, yellow fever group). Nineteen of them are tick-borne viruses, which are sub-divided into the mammalian group and a seabird group, and TBEV is classified as one of the species within the mammalian group (Heinz et al., 2000). The remaining viruses are zoonotic agents without any known vector (Entebbe bat virus group, Modoc virus group, Rio Bravo virus group) (Monath and Heinz, 1996). The most important human pathogens are yellow fever virus, Japanese encephalitis virus, the dengue viruses, West Nile virus, and TBEV (Rice, 1996).

TBEV is subdivided into the European (TBEV-Eur, Western), the Far-Eastern (TBEV-FE) and the Siberian (TBEV-Sib) subtypes, respectively.

The TBE virion is a spherical particle with a diameter of about 50 nm. It contains a capsid composed of a positive-stranded RNA genome and the spherical nucleocapsid protein C, which is surrounded by the viral envelope. The lipid envelope carries the major envelope glycoprotein E with a small membrane associated protein, M (Rice, 1996). The E glycoprotein is the major viral antigen that induces neutralizing antibodies and a protective immune response. It interacts with cell receptors and mediates low pH-triggered membrane fusion in the endosome. X-ray crystallography has shown that E protein molecules are oriented parallel to the virus membrane and do not form spiky projections. The basic subunit of the virion envelope is a dimer consisting of two protein E molecules with antiparallel orientation (Rey et al., 1995). The M protein is present as a precursor (prM) in immature virions and forms a heterodimeric complex with the E glycoprotein that protects the E glycoprotein from undergoing conformational changes in the acidic environment (Heinz et al., 1994). Before exocytosis the prM-protein is cleaved by furin and the E-M interaction is destabilized which makes the virion mature and infection (Stadler et al., 1997) (Fig. 1).

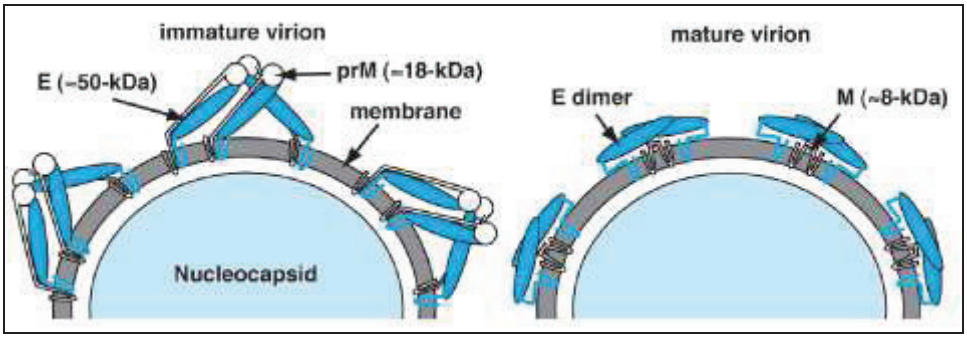

Figure 1. Schematic of TBEV particle in its immature and mature form (Lindenbach et al., 2007).

1.1.1. Genomic organization of TBEV

The TBEV genome is single positive-stranded RNA molecule of approximately 11 kb, with a type I 5' cap, $m^7GpppAmpN_2$ (Cleaves and Dubin, 1979) and lacking 3' polyadenylation. Like in all positive RNA viruses, the flavivirus genome serves as mRNA for the initial translation of viral proteins. The genome encodes a single long open reading frame (ORF) of about 3400 amino acids (aa), which is cleaved co- and post-translationally into 10 proteins: three structural proteins (C, M and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). The open reading frame is flanked by 5' and 3' noncoding regions (NCR) of ≈100 nucleotides (nt) and 400 to 700 nt, respectively.

The 5'- and 3'- noncoding regions of the genome are of great importance for initiation and regulation of translation, replication and virion assembly (Proutski et al., 1997; Rauscher et al., 1997). The 5'NCR of TBEV contains an 11 nt-long conserved motif that is located approximately 100 nt from the 5'-terminus, and represents a perfect inverted repeat of another conserved motif located approximately 80 nt from the 3'-terminus. These motifs probably play a role in genome circulation. Computer prediction of the 5'NCR secondary structure revealed a stem-loop pattern, which is shared by all viruses of the TBEV complex (Mandl et al., 1993; Gritsun et al., 1997). The 3'NCR is of variable length among TBEV strains, and some of them contain a poly A-tract (Wallner et al., 1995). The 3'NCR can be divided into a 3'-terminal core element of approximately 340 nt and a variable region located between the core element and the ORF. The core elements are present in all strains investigated to date, and their sequences are highly conserved among the strains. The entire core element is predicted to fold into a well-defined secondary structure (Rauscher et al., 1997). The 3'-terminal sequences and secondary structures are proposed to act as viral promoters for initiation of minus-strand RNA synthesis, and more proximal sequences and structures as enhancers of viral RNA replications.

Deletions or mutations in these regions influence the functional properties of the virus and its virulence (Mandl et al., 1998).

1.1.2. TBEV proteins

1.1.2.1. Structural proteins

The nucleocapsid protein (C) is a highly basic protein with a molecular weight of approximately 11 kDa. Charged residues are clustered at the N- and C-termini which presumably mediate the RNA interaction (Khromykh and Westaway, 1996). Nascent C protein (anch C) contains a C-terminal hydrophobic anchor that serves as a signal peptide for ER translocation of prM. The hydrophobic domain is cleaved from mature C by the viral serine protease (Yamshchikov and Compans, 1994).

The membrane precursor of the M protein (prM), a glycoprotein with a molecular weight of approximately 26 kDa, is translocated into the ER by the C-terminal hydrophobic domain of C protein, contains 12 conserved cysteine residues. A major of prM protein is to prevent E from undergoing acid-catalyzed rearrangement into the fusogenic form during transit through the secretory pathway. The conversion of immature virus to mature virions occurs in the secretory pathway and coincides with cleavage of prM into pr and M fragments by the Golgi-resident protease furin (Mandl et al., 2000).

The envelope protein (E) is the major protein on the surface of the virus with a molecular weight of approximately 53 kDa. E is synthesized as a type I membrane protein containing 12 conserved cysteines that form disulfide bonds and the N-glycosylation site at position Asn 155 (Mandl et al., 1988). The E proteins form dimers on the virion surface, and associate in a head-to-tail arrangement with an antiparallel orientation (Rey et al., 1995). Each of the protein monomers is composed of three distinct domains designated I-III. Domain I is formed by the N-terminal part of the protein; it has a beta barrel structure, two disulfide bridges and carries the single carbohydrate side chain. Domain II is composed of two independent discontinuous long loops and one of them is stabilized by three disulfide bridges and forms the tip that functions as an internal fusion peptide. Domain III includes the C terminal part of the protein and has the typical fold of an immunoglobulin constant domain. It is thought to be a receptor-binding region (Rey et al., 1995; Mandl et al., 2000). The upper surfaces of the ectodomains carry the antigenic determinants recognized by neutralizing antibodies. Sequence analysis of the E proteins of all available TBEV stains revealed several “signature” aa residues, which are unique for each subtype of TBEV (Ecker et al., 1999).

1.1.2.2. Non-structural proteins

The NS1 protein (approximately 46 kDa) is associated with membranes as hexameric ring-like particles ~10 nm in diameter (Gritsun et al., 1988; Gritsun et

al., 1990; Flamand et al., 1999). This protein induces protective immune responses and is called “soluble antigen”.

The NS2A protein (approximately 22 kDa) is a hydrophobic protein, that is probably involved in coordinating flavivirus RNA replication and packaging (Khromykh et al., 2001).

The NS2B is a small (approximately 14 kDa) membrane-associated protein that forms a complex with NS3 acts as a cofactor for the NS2B-NS3 serine protease (Chambers et al., 1991).

The NS3 (approximately 70 kDa) is a large protein with several enzymatic activities required for polyprotein processing and RNA replication. The N-terminal third of the protein is the catalytic domain of the NS2B-NS3 serine protease complex (Gorbalenya et al., 1989; Chambers et al., 1993). The C-terminal region of NS3 encodes regions of significant homology to supergroup 2 RNA helicases. RNA-stimulated nucleoside triphosphatase (NTPase) activity (Wengler, 1993) and RNA unwinding (helicase) activity have been confirmed an essential roles for viral replication (Gorbalenya et al., 1989).

NS4A and NS4B (16 kDa and 27 kDa, respectively) are hydrophobic transmembrane proteins. A role for NS4A in RNA replication is supported by the co-localization of this protein with replication complexes. The function of NS4B remains unclear.

The NS5 (103 kDa) is a highly conserved multifunctional protein with methyltransferase (MTase) activity, which involves in N-terminal RNA cap-processing and C-terminal RNA-dependent RNA polymerase (RdRP) activity. The NS5 protein also blocks signalling of IFN-α/β and INF-γ by binding to their receptors (Rice et al., 1985; Koonin, 1993).

1.1.3. Life cycle of TBEV

The virus infects susceptible cells via receptor-mediated endocytosis. Flaviviruses can utilize multiple receptors for different cell types and in different host species. Recent investigations provided evidence for virion binding to the surface glycosaminoglycans as an important role in the initial attachment of several flaviviruses to target cells (Mandl et al., 2001; Mandl, 2005). After binding to cellular receptors, the virions are internalized via clathrin-coated pits and trafficked to a pre-lysosomal endocytic compartment, where low pH triggers a structural alteration in the E protein, that in turn leads to the fusion of the viral and endosomal membranes and release of the nucleocapsid (Gollins and Porterfield, 1985, 1986; Heinz et al., 1994). In the mature virion, the E protein exists as a metastable dimer, that dissociates at the acidic pH in endosomes and is converted into a more stable trimeric conformation. The dimer dissociation step liberates an internal peptide that interacts with the target endosomal membrane, and then further conformational changes are believed to drive the membrane fusion (Bressanelli et al., 2004; Heinz et al., 2004).

The viral genome is released into the host cytoplasm by a process of nucleocapsid uncoating. Replication begins with the synthesis of a genome negative strand complementary RNA, which then serves as a template for the synthesis of additional positive strand RNA. Negative strand RNA has been detected as early as 3 hours after infection (Heinz et al., 1994). Viral RNA synthesis is asymmetric, with positive strands accumulating in around tenfold excess over negative strands. Translation of the viral genome produces proteins that lead to replication of the viral genome and assembly of new virus particles. It has been shown that genome packing is coupled to RNA replication (Khromykh et al., 2001).

Virus assembly takes place in the endoplasmic reticulum (ER) and first leads to the generation of fusion-incompetent, immature virions, in which the E protein forms a stable heterodimeric complex with the precursor of the M protein (prM) (Allison et al., 1995). It has been shown that co-expression with prM is essential for proper maturation, transport and secretion of the E protein (Konishi and Mason, 1993; Allison et al., 1995), and it has been suggested that prM also plays a chaperone-like role in the folding of the E protein (Lorenz et al., 2002). The maturation cleavage of prM is apparently mediated by furin (Stadler et al., 1997) and occurs in the trans-Golgi network shortly before the release of mature virions via budding at the plasma membrane.

1.1.4. TBEV, vectors and reservoirs

Two tick species, *I. ricinus* and *I. persulcatus*, are the main vectors for TBEV. They belong to the hard tick family (*Ixodidae*), named after their scutum, i.e. the shield covering parts of their dorsal body surface. The duration of their life cycle varies from two to six years and involves four different stages of development: egg, larva, nymph and adult. All ticks feed only once per stage and the tick's life span depends on the time intervals between successful feedings as well as climatic conditions. Nymphs and larvae are the most important stages in the transmission of TBEV as they are more numerous than adults (Suss, 2003). A variety of host animals has been described for *Ixodes* ticks with more than 300 different species of wild and domestic mammals, birds and reptiles. The most important hosts for immature ticks (nymphs and larvae) and reservoirs for TBEV are wild rodents, mainly of the genera *Apodemus* and *Myodes* that act as reservoir hosts (Kocianova et al., 1993; Labuda et al., 1993). Larger animals act as reproductive or amplifying hosts for the adult ticks, but are considered to be incompetent in terms of virus transmission (Gerth et al., 1995). In general, the immature stages, larvae and nymphs, feed on small mammals and birds, and adults on larger animals. Larger animals, e.g. deer, cows, and sheep become infected, but levels of viraemia may be low and of short duration.

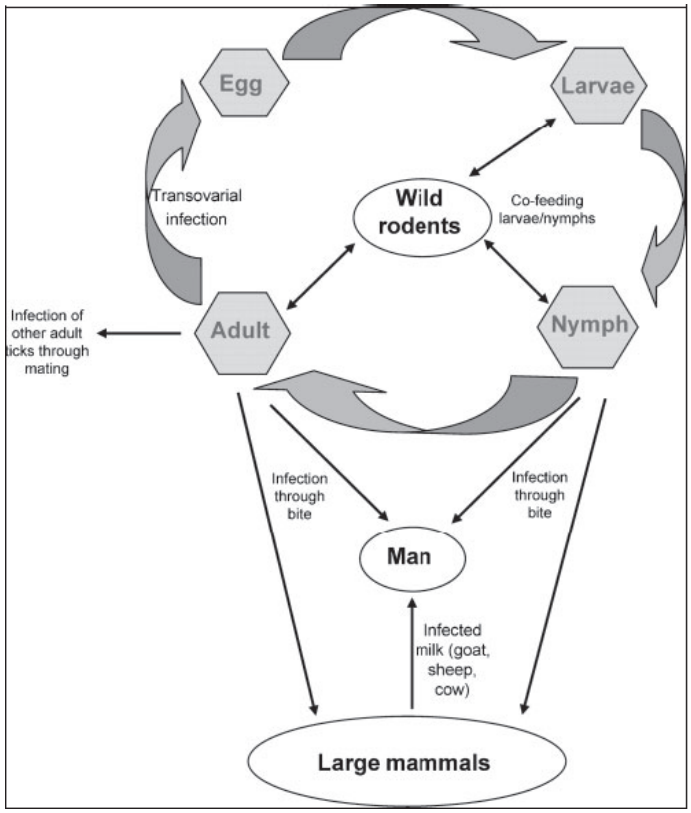

Figure 2. Transmission of TBEV within the life cycle of *I. ricinus* (Mansfield et al., 2009).

Ticks may be infected with TBEV at different stages of their development during co-feeding on small animals, and remain infected during the rest of their lives. Infected ticks thus transmit virus to non-infected ticks during co-feeding at the same site on the host. Replication of TBE virus begins during a blood meal after entry into the wall cells of the midgut. After infection of midgut cell, virus passes to the salivary glands. Transmission of virus from ticks to a vertebrate host during feeding occurs via tick saliva that contains virus secreted from infected salivary gland cells (Nuttall et al., 1994). As previously reported, co-feeding is the major factor in virus transmission, where vertebrate hosts can play an important role in TBEV transmission in the absence of a detectable level of viraemia and in the presence of detectable levels of neutralizing antibodies. During co-feeding, virus transmission occurs via cellular infiltration of tick feeding sites and the migration of cells from these sites (Labuda et al., 1996). Transovarial transmission also can occur, while only few larvae get infected from eggs of infected female ticks (<0.5%) (Danielova and Holubova, 1991) (Fig.2).

1.1.5. Distribution and molecular epidemiology of TBEV

As mentioned, *Ixodes* ticks are more associated with TBE virus. The ticks are relatively immobile, and move almost exclusively vertically on the vegetation in their quest for suitable animals. These animals, in turn, inhabit a very local territory and therefore the virus is spread rather slowly across the forest floor. Consequently the ticks, and the vertebrate hosts play central roles in the spread and evolution of the tick-borne viruses.

The geographical distribution of *I. ricinus* includes large areas of Europe and parts of North Africa. *I. presulcatus* has a vast range in Eurasia, from the Baltic countries Estonia and Latvia in the west to northern Japan in the east. Natural TBE foci coincide with the distribution of these vector ticks.

Based on phylogenetic analyses, three subtypes of TBEV have been described, the European (TBEV-Eur, western), transmitted by *I. ricinus*, the Far-Eastern (TBEV-FE) and the Siberian (TBEV-Sib) subtypes transmitted by *I. persulcatus* (Ecker et al., 1999). The TBEV-Eur subtype is widely distributed across Europe with strains having been isolated in Austria, Switzerland, Sweden, Germany, Hungary, Czech Republic, Slovenia, Finland, Latvia, Lithuania, Estonia, Belarus, and the European part of Russia (Ecker et al., 1999; Lundkvist et al., 2001; Haglund et al., 2003). TBEV-FE and TBEV-Sib strains are spread from Japan and Far East Russia to the Baltic-Nordic countries, Latvia, Estonia and Finland (Mavtchoutko et al., 2000; Lundkvist et al., 2001; Mickiene et al., 2001; Jaaskelainen et al., 2006).

Recent studies have shown that the TBEV-Sib and TBEV-FE subtypes are phylogenetically more closely related to each other than to the TBEV-Eur subtype (Grard et al., 2007). In our previous study, we found that TBEV is widely distributed in Estonia and all three subtypes have been isolated from ticks and rodents. Phylogenetic analyses showed that Estonian and Latvian TBEV strains formed own lineage within TBEV-Sib subtype (Golovljova et al., 2004).

1.2. *Anaplasma phagocytophilum*

Anaplasma phagocytophilum, a species in the genus *Anaplasma*, is known as an emerging tick-borne pathogen of medical and veterinary importance in Europe and in North America. This pathogen is a gram-negative, obligate intracellular bacterium, that infects granulocytes and multiplies within membrane-bound vacuoles in the cell cytoplasm, causing an acute, nonspecific febrile illness in a wide range of host mammals and, as more recently described, also in humans. *A. phagocytophilum* is the causative agent of human granulocytic anaplasmosis (HGA), “tick-borne fever” (TBF) of ruminants and equine and canine granulocytic anaplasmosis (Dumler et al., 2001).

In 1994 the first case of human granulocytic anaplasmosis was reported in the United States (Chen et al., 1994), and since then, the number of clinical cases of HGA has been steadily increasing. As of 2010, the number of cases has increased to 2963 in USA, and HGA has become one of the most common tick-borne pathogens in the United States (Doudier et al., 2010).

The first confirmed case of HGA in Europe was described in Slovenia in 1996 (Petrovec et al., 1997) and the number of reported human cases is continuously increasing. HGA has been reported in the Netherlands, Spain, Poland, Croatia, Sweden, Norway, Austria, Italy (Blanco and Oteo, 2002; Parola et al., 2005), France (Remy et al., 2003) and the far east of Russia (Sidel'nikov Iu et al., 2003). According to this data the number of cases in Europe was nearly 70 (Dumler et al., 2005). The case fatality rate in humans is less than 1% (Walker and Dumler, 1997). Clinically, HGA presented as as moderately severe febrile illness with headache, myalgia, malaise, and reported laboratory findings included leucopenia, thrombocytopenia and evidence of hepatic injury. Although the disease is usually self-limiting, severe complications may occur; these include prolonged fever, shock, seizures, pneumonitis, acute renal failure, hemorrhage, rhabdomyolysis, opportunistic infections and death (Carlyon and Fikrig, 2003). Human cases have a seasonal distribution and occur mainly during summer, when ticks are most active. No vaccines are available for this disease, and doxycycline, a broad-spectrum antibiotic, remains the drug of choice for treating patients (Rikihisa and Lin, 2010).

Clinical signs of tick-borne fever in ruminants (goats, sheep, cattle) differ from no detectable illness to severe febrile disease associated with opportunistic infections, haemorrhage and abortions. TBF was reported in the Netherlands, UK, Scandinavia, Spain, Switzerland, France and Germany (Dumler et al., 2001).

In general, several factors such as the variants of *A. phagocytophilum* involved, co-infection with other pathogens, immune status, condition, and age of the host, as well as factors like climate may influence the severity of the infection (Stuen, 2007).

1.2.1. Genomic organization

Anaplasma phagocytophilum has a single circular chromosome. Its genome size is approximately 1494 kbp. Genes responsible for cell wall biosynthesis are not found in these species, which does not synthesize peptidoglycan. The genome has about 462 open reading frames (ORFs) (Rydkina et al., 1999).

Genetically *A. phagocytophilum* has been characterized by the analysis of small subunits of rRNA (16S rRNA), a heat-shock protein (*groESL*) and the AnkA protein (*ankA*) genes. Genes which encode the 16S rRNA and *groESL* heat-shock protein are highly conserved in bacteria and are often used in phylogeny for taxonomic classification, and in differentiation and identification

of species. Within the species, *A. phagocytophilum* 16S rRNA gene sequences show more than 99.5 % identity and *groESL* sequences show around 99.0% identity (Dumler et al., 2001).

Although the similarities between the 16S rRNA gene sequences are high, several genetic variants with differences in the variable region near the 5'end of the 16S rRNA gene at position 76 to 84 have been found (Chae et al., 2000; Massung et al., 2002; Stuen et al., 2002).

According to phylogenetic analysis based on a gene *groESL* two genetic lineages of *groESL* sequences were found (Petrovec et al., 2002; von Loewenich et al., 2003). The first one includes the wide spectrum of different reservoir hosts like red deer, sheep, horses, *I. ricinus* ticks as well as humans, and widely distributed in Europe and the USA, while the second lineage consists of exclusively narrow range of two species such as roe deer and *I. ricinus* tick and detected only in Europe (von Loewenich et al., 2003; Katargina et al., 2011).

The third gene, which has been actively studied, is *ankA.* This gene contains several ankyrin repeats that are thought to mediate protein-protein interaction, but the exact function has not been determined yet (Rikihisa and Lin, 2010). It is more variable than the highly conserved 16S RNA and *groESL.* According to genetic analysis, several different patterns of ankA gene organisation have been found (Massung et al., 2000; von Loewenich et al., 2003; Scharf et al., 2011). Based on the respective *groESL* and the *ankA* sequences, the strains of *A. phagocytophilum* formed similar lineages on the phylogenetic trees (von Loewenich et al., 2003).

1.2.2. Morphology

Anaplasma phagocytophilum is a gram-negative, small, often pleomorphic, coccoid to ellipsoidal organism that resides within cytoplasmatic vacuoles, either singly, or more often in compact inclusions (morulae). It is present in mature or immature haematopoietic cells, particularly myeloid cells and neutrophils, as well as erythrocytes, in peripheral blood or in tissue, usually in mononuclear phagocyte organs (spleen, liver, bone marrow) of the mammalian host (Fig.3).

In mammalian cells, morulae are usually 1.5-2.5 μm in diameter, but may be as large as 6 μm. Morphologically two forms were observed: larger reticulate cells and smaller forms with condensed protoplasm called dense-core forms (Popov et al., 1998). Individual bacterial cells are of two types, and both occur in the same vacuole and may undergo binary fission.

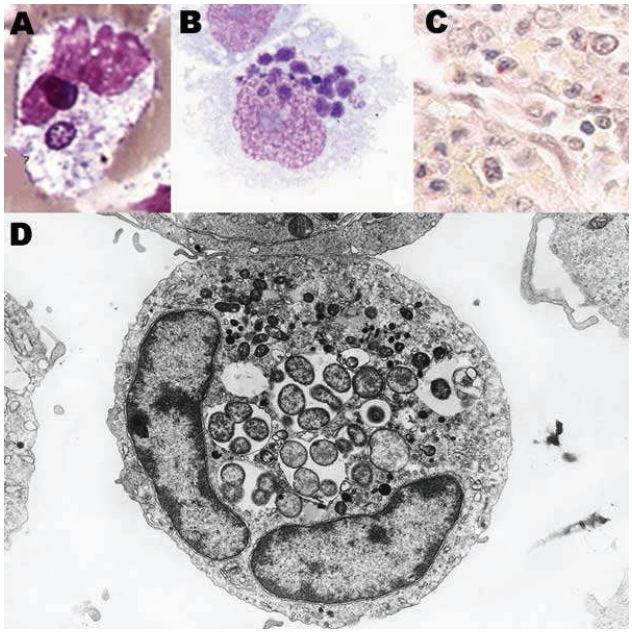

Figure 3. *Anaplasma phagocytophilum* in human peripheral blood band neutrophil (A), in THP-1 myelomonocytic cell culture (B), in neutrophils infiltrating human spleen (C), ultrastructure by transmission elektron microscopy in HL-60 cell culture (Dumler et al., 2005).

1.2.3. Ecology and transmission

A. phagocytophilum infects a wide range of animals including rodents, ruminants, felids, horses, donkeys, and dogs as well as humans.

The ecology of *A. phagocytophilum* involves ticks as vectors and vertebrates as reservoir hosts.

Ticks of the *Ixodes* species play an important role as vectors in the transmission and maintenance of *A. phagocytophilum* in natural foci. Several tick species are widely spread in the following regions: *I. scapularis* in the eastern United States, *I. pacificus* in the western United States (Doudier et al., 2010), *I. ricinus* in Europe (Strle, 2004) and *I. persulcatus* in the Russian Baltic region (Alekseev et al., 1998) and in the Urals, Siberia, the Far East (Rar et al., 2010b).

Roe deer (*Capreolus capreolus*) are the natural host reservoirs of *A. phagocytophilum* in Europe use (Petrovec et al., 2002), as are the rodents yellow-necked mouse (*Apodemus flavicollis*) and bank vole (*Clethrionomys gareolus*) (Liz et al., 2000). while the white-footed mouse (*Peromyscus leucopus*) and the white-tailed deer (*Odocoileus vinginianus*) are hosts in North America (Dumler et al., 2005). The wide and expanding host range of *A.*

phagocytophilum most likely ensures an increasing distribution of pathogens worldwide and the continued emergence of granulocytic anaplasmosis in domestic animals and humans. These vertebrate hosts develop persistent infections, which in its turn, allows them to serve as reservoirs of infection and key hosts by feeding both immature and mature stages of *Ixodes* ticks, thus modulating local tick abundance and the occurrence of *Anaplasma* infection (Kocan et al., 2008).

Migrating birds have been considered to be important in the dispersal of *A. phagocytophilum* infected *I. ricinus* and in the distribution of granulocytic anaplasmosis in Europe (Kocan et al., 2008). Humans are infected accidentally by the bite of infected ticks. Human-to-human nosocomial transmission of *A. phagocytophilum* may occur through direct contact with blood or respiratory secretions (Zhang et al., 2008).

A. phagocytophilum is transmitted horizontally (transstadially)by ticks, while vertical (transovarial) transmission does not occur; thus emergent larval ticks are not infectious (Dumler et al., 2001).

The developmental cycle of *A. phagocytophilum* in ticks has not been properly studied. More extensive infection processes during the tick feeding cycle were described for another of *Anaplasma* species, i.e. *A. marginale.* During the blood meal ticks ingest bovine erythrocytes containing the pathogen. The midgut cell is the first site of infection in ticks, where the first replication of the pathogen occurs. Following transport to the salivary glands, the bacteria penetrate into epithelial cells. After attachment and in during the feeding process on a subsequent host, the pathogen undergoes a second cycle of replication in the salivary gland, and is then excreted in the saliva (Ueti et al., 2009). In addition to midgut and salivary gland sites, other tick tissue may also become infected (de la Fuente et al., 2009).

1.3. *Babesia* species

Babesia species (Apicomplexa, Piroplasmida) are protozoan parasites of domestic and wild animals. All babesia parasites are transmitted by *Ixodes* ticks to their vertebrate hosts. The parasites replicate in the hosts' red blood cells and are called piroplasms due to their pear-shaped appearance within the infected host cells (Fig. 4) (Telford, 1993). *Babesiae* infect mammals, i.e. cattle, sheep, goats, horses, pigs, dogs and cats and occasionally man, and cause a hemolytic disease known as babesiosis. The parasites are named after the Romanian scientist Victor Babes, who first described and identified *Babesia* species in cattle in 1888. A few years later Smith and Kilbourne demonstrated ticks as the vector for *Babesia* (1893). The first recognized case of human babesiosis caused by the cattle parasite *B. divergens* was documented in Yugoslavia (Skrabalo and Deanovic, 1957). Since then, about 40 cases caused by *B. divergens* have been reported in Europe (Grey et al., 2010). Three different babesia parasites have

been recognized as the primary agents of human disease: the cattle species *B. divergens* and *Babesia* sp. EU1 in Europe (Gorenflot et al., 1998; Herwaldt et al., 2003; Haselbarth et al., 2007) and the rodent species *B. microti* in North America (Homer et al., 2000; Hunfeld and Brade, 2004). The tick vector in western Europe is believed to be the European sheep tick *I. ricinus* - one of the most commonly encountered ticks in central and western Europe, while *I. persulcatus* is considered to be the vector in the eastern part of Europe and Asia (Rar et al., 2010a). In the North America the main tick-vector of babesiae is *I. scapularis.*

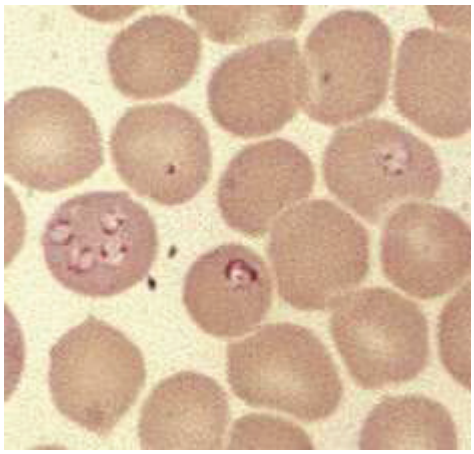

Figure 4. The blood smear of a patient infected by *B. microti*, seen as rings within the blood cells.

1.3.1. Classification of *Babesia* species

The taxonomic classification of *Babesia* species places them in the phylum *Apicomplexa*, class *Aconoidasida* and the other *Piroplasmida*. Two of the families within the other *Piroplasmida* are *Babesiidae* and *Theileriidae*. At least 100 species of tick-transmitted *Babesia* have been described. They are associated with various domestic and wild animals, and the tick species acting as vectors have been identified. Description and classification of *Babesia* species are based on morphological and genetical characteristics. *Babesia* are grouped into the small *Babesia* with tropozoite sizes of 1.0 to 2.5 μm (*B. gibsoni*, *B. microti*, *B. rodhaini, B. spp* WA1 and et.c.) and large *Babesia* species with with tropozoite sizes of about 2.5 to 5.0 μm (*B. bovis*, *B. caballi*, *B. canis, B. odocoilei*, *Babesia* sp. EU1 etc.*)*. These morphological classifications are generally consistent with the phylogenetic characterization based on 18S rRNA, which shows that the large and small babesias fall into two phylogenetic clusters (Homer et al., 2000). One exception to this is *B.divergens;* morphologically this

pathogen is small with a trophozite size of about 0.4 to 1.5 µm, but genetically it is related to large *Babesia* species.

The three primary *Babesia* species, members of both the "small" and the "large" group which have been found to infect humans are *B. microti*, *B. divergens* and *Babesia* sp. EU1, respectively. They have different host requirements and the disease manifestations are somewhat different.

1.3.1.1. Babesia microti

B. microti, the rodent borne *Babesia,* is recognized as the etiologic agent of human babesiosis (also known as "Nantucket fever") in the United States and is reported to infect humans in Germany (Telford, 1993; Kjemtrup and Conrad, 2000; Hildebrandt et al., 2007). This parasite is endemic, but non-pathogenic in mice populations and vectored by the *Ixodes* ticks (Gorenflot et al., 1998). It is well established that *Ixodes scapularis* in the USA and *I. ricinus* in Europe are vectors of *B. microti* to a wide variety of host species, including humans and domestic animals e.g. dogs (Foppa et al., 2002; Gray, 2002; Welc-Faleciak et al., 2007). In North Ural and West Siberia *B. microti* was detected in *I. persulcatus* (Rar et al., 2010a).

According to phylogenetic analysis based on the comparison of the 18S rRNA and beta-tubulin genes of *B. microti* from different countries and different vertebrate hosts several clusters of species were recognized: "US"-type, isolated from microtine rodents in United States and different parts of Eurasia (Zamoto et al., 2004; Grey et al., 2010), "Munich"-type, isolated from rodents and ticks in Germany (Pieniazek et al., 2006; Sinski et al., 2006; Nakajima et al., 2009), and "Kobe"-type and "Hobetsu"-type isolated from humans and rodents in Japan (Tsuji et al., 2001; Wei et al., 2001).

The first human case of *B. microti* infection in North America was detected in 1968. Since then, hundreds of cases have been reported, with approximately 40 resulting from blood transfusions and transplacental transmission (Lux et al., 2003). Most of the cases are tick-transmitted and occur in spleen-intact individuals. Humans with *B. microti* infections commonly exhibit a flu-like presentation including fever, malaise and myalgia, but they have also been reported to be asymptomatic, since babesiosis is self-limiting and mild in most patients (Gorenflot et al., 1998). The fatality rate is about 5% (Hunfeld et al., 2008). The first European human case caused by *B. microti* was reported in Germany (Hildebrandt et al., 2007) and a wide distribution of this pathogen in ticks and small mammals has been demonstrated in Europe (Duh et al., 2001; Skotarczak and Cichocka, 2001; Foppa et al., 2002; Goethert and Telford, 2003; Kalman et al., 2003).

Morphologically, this organism is classified as pleomorphic and can be seen predominantly as rings, but also as pairs and tetrads, in vertebrate blood. The *B. microti* tetrad formation is distinct from that of *B. divergens* in that it is the result

of the quaternary budding of one organism into four, rather than the binary fission of one organism into two, as seen with *B. divergens* (Levine, 1985).

1.3.1.2. Babesia divergens

B. divergens is an important pathogen of cattle (Duh et al., 2001; Hartelt et al., 2004; Casati et al., 2006) and the main causal agent of human babesiosis in Europe (Grey et al., 2010). Geographic overlap of *I. ricinus* tick populations, infected cattle populations, and the origin of human cases, strongly implicates the *I. ricinus* tick as the responsible vector to humans in Europe. In the Russian Novosibirsk region, *B. divergens* was found also in *I. persulcatus* ticks (Rar et al., 2010b). The presence of *B. divergens* parasites in naturally infected roe deer has been recently reported in Slovenia (Duh et al., 2005), Poland (Sawczuk et al., 2005), Spain (Garcia-Sanmartin et al., 2007), Switzerland (Hoby et al., 2007) and Italy (Tampieri et al., 2008).

Human cases occur most often in rural areas where cattle are present and correspond to the seasonal activity of the tick vector. Eighty three percent of the documented European patients have been asplenic, and have a high (42%)fatality rate (Gorenflot et al., 1998). The broad range of symptoms, which include fever, fatigue, chills, hemoglobinuria, thrombocytopenia, hepatomegaly, and splenomegaly, are the result of the parasite-induced hemolytic anemia (Homer et al., 2000).

Morphologically, *B. divergens* is a small parasite most commonly seen in pairs, which diverge widely from each other near the circumference of the infected bovine erythrocyte, but can also be seen as single round parasites and rarely as tetrads (Levine, 1985). In human erythrocytes, the pairs are seen in central or subcentral portions of the cell (Gorenflot et al., 1998). *B. divergens* has one of the largest host ranges described to date for a *Babesia* species (Zintl et al., 2003). It is able to infect splenectomised primates (chimpanzees, rhesus monkeys) (Garnham and Bray, 1959), ungulates (roe deer, fallow deer, red deer, mouflon and sheep) (Enigk and Friedhoff, 1962) and rodents (rats) (Phillips, 1984) as well as non-splenectomized reindeer (Nilsson et al., 1965), sheep (Malandrin et al., 2010) and gerbils (Lewis and Williams, 1979).

According to phylogenetic analysis of complete 18S rRNA, *B. divergens* isolates are closely related (99.83% identity) to *Babesia capreoli* isolates with a clear distinction at three constant positions (nucleotides: 631, 663, 1637) (Malandrin et al., 2010). These two species could not be clearly differentiated on the basis of morphology or serology (Gray et al., 1990). Also *B. caprioli* differed from *B. divergens* by the fact that it did not infect splenectomised cattle or gerbils (Gray et al., 1990). However, the distinction between them remains uncertain, and their status as different species is sometimes questioned (Gray, 2004; Duh et al., 2005), which is why *B. capreoli* occasionally is designated – "*B. divergens* – like".

1.3.1.3. Babesia sp. EU1

Another one novel non - *B. divergens* species, EU1 agent, is responsible for three cases of human babesiosis in Germany, Italy and Austria, respectively (Herwaldt et al., 2003; Haselbarth et al., 2007). *Babesia* sp. EU1 is transmitted by *Ixodes ricinus* ticks and was identified in blood from a natural host (roe deer) in Slovenia (Duh et al., 2005), France (Bonnet et al., 2007) and Italy (Tampieri et al., 2008).

Clinical manifestations and symptoms of the disease include elevated body temperature, chills, anaemia, weakness, fatique, anorexia, and headache, followed by jaundice and dark urine from haemoglobinuria. Delay of treatment can be fatal, since human babesiosis may be further complicated by central nervous system involvement, or severe complications, such as congestive heart failure, intravascular coagulation, renal failure, and respiratory distress syndrome, if left untreated (Homer et al., 2000).

According to phylogenetic analysis, EU1 clusters together with *B. odocoilei*, and these two organisms form a sister group with *B. divergens* (Herwaldt et al., 2003). EU1 differs from *B. odocoilei* and *B. divergens* by 29 and 31bases of the 18S rRNA gene, respectively. Serologic and phylogenetic analyses distinguish this organism from other known human *Babesia* agents (Herwaldt et al., 2003).

1.3.2. Biology of *Babesia*

The life cycle of Babesia begins when the infected tick injects the parasite through the saliva into the vertebrate host's blood while feeding (Fig. 5). *Babesia* enters erythrocytes at the sporozoite stage. *Babesia* sporozoites penetrate directly into the red blood cells and all parasitic stages (merozoites, gametocytes) develop in the red blood cells. The life cycle includes asexual multiplication in vertebrate blood cells, sexual reproduction in the tick vector, and the production of sporozoites in the salivary glands of the vector (Mehlhorn and Shein, 1984; Telford, 1993).

Parasite maintenance and persistence within the tick vector is ensured by transovarial and transstadial transmission, depending on the *Babesia* species (Kjemtrup and Conrad, 2000; Chauvin et al., 2009). Transovarial transmission of *B. divergens* occurs when developing parasites invade the ovaries of the tick vector and infect the oocytes within. The infected eggs hatch, the larval ticks mature and are then capable of transmitting the parasites during feeding without having previously ingested an infected blood meal. Transovarial transmission is an efficient method of parasite survival in that thousands of developing ticks can become infected while in the ovary of the female tick (Telford, 1993). *B. microti* does not undergo transovarial transmission, but once a larva has become infected by feeding on an infected mammalian host, it is able to pass on the infection transstadialy to the nymph (Levine, 1985), which remains infective through the following molt.

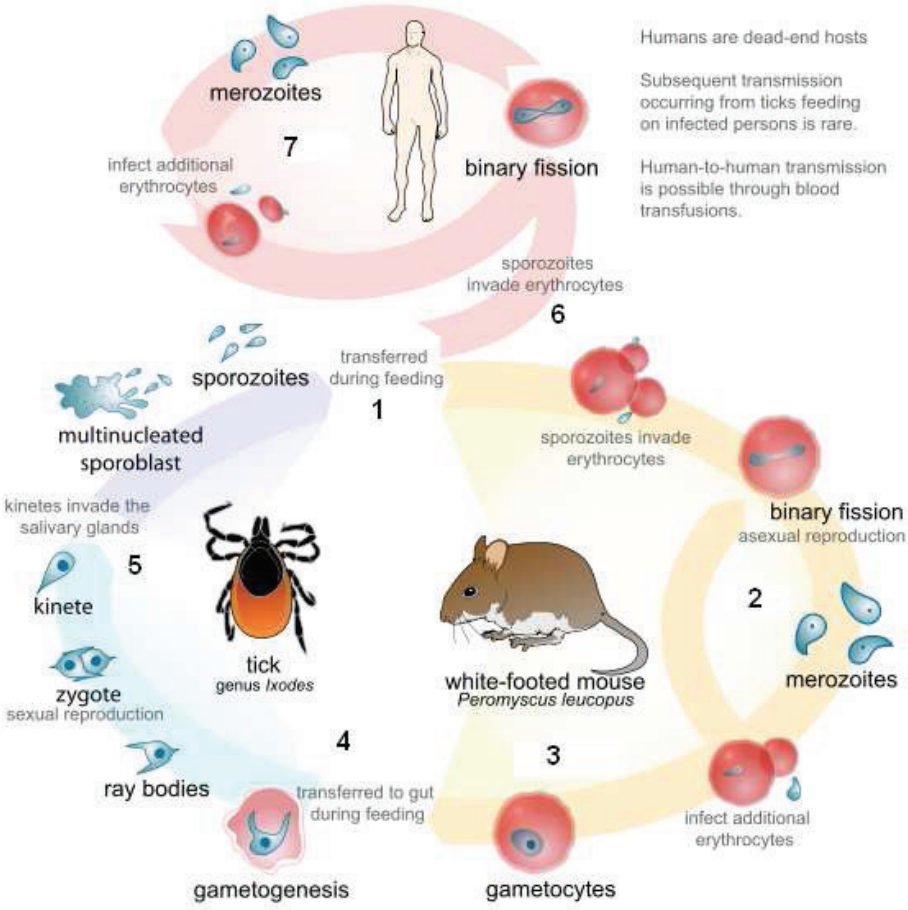

Figure 5. The life cycle of *B. microti* parasite fluctuates principally between the tick and rodent hosts. 1) During feeding, a *Babesia*-infected tick introduces sporozoites into erythrocytes of the vertebrate host, i.e. a mouse or another small mammal. 2) The *Babesia* parasites undergo an asexual reproductive stage with transformation into merozoites and trophozoites and eventually (3) gametocytes. Since asexual reproduction can occur within white-footed mice, they act as reservoirs for babesia. 4) The definitive host is the tick, where the *Babesia* parasite can then undergo sexual reproduction. 6) Humans enter the cycle when bitten by infected ticks. Sporozoites traffic to the erythrocytes, where infection occurs
(http://en.wikipedia.org/wiki/File:Babesia_life_cycle_human_en.svg).

2. AIMS OF THE STUDY

The general objective of this study was to improve our knowledge about tick-borne pathogens (TBP) circulating in Estonia, their presence, distribution and prevalence rates.

This was approached through the following specific aims:

1. To analyze TBEV strains of the Siberian subtype circulating in Estonia.
2. To investigate the presence and prevalence of *A. phagocytophilum* in tick populations in Estonia and to provide a genetic characterization and phylogenetic analysis of local *A. phagocytophilum* strains.
3. To investigate the presence and prevalence of *Babesia* spp. in tick populations in Estonia and to provide a genetic characterization and phylogenetic analysis of local *Babesia* species.

3. MATERIALS AND METHODS

3.1. Collection of ticks

Tick sampling from the vegetation was performed by flagging from April to November during 2002-2008 at 14 sites in mainland Estonia, 10 sites on Saaremaa island, four sites in the European part of Russia (St. Petersburg area, Curonian Spit, Borok and Tcherepovets) and at 7 sites in Belarus (Minsk area). Collected ticks were pooled into groups according to collecting sites, stages and sex, or individually, washed in 70% ethanol and rinsed twice with sterile PBS. Tick pools or individual ticks were homogenized and suspended in 400 µl of PBS. Suspensions were stored at -70ºC for future RNA/DNA extraction.

3.2. RNA/DNA extraction

RNA/DNA was extracted from 200 µl tick suspension with the guanidinium thiocyanate-phenol-chloroform method using the TriPure isolation system (Roche Diagnostics, Lewes, UK) according to the manufacturer's instructions.

3.3. PCR amplification of the partial E-protein gene of TBEV RNA

Amplification of the viral genome encoding the E protein was performed as described earlier (Mickiene et al., 2001). Briefly, the partial E-protein gene of TBEV was amplified with forward primer 41F1 and reverse 1312R (Table 1). RT-PCR amplification was carried out in a total reaction volume of 25 µl, which contained the following mix of reagents: 2x Reaction mix (Invitrogen), 0.2 µM of forward and reverse primers, 1 µl of SuperScript® III RT/ Platinum® *Taq* Mix (Invitrogen), and 2.5 µl of target RNA. The cycling conditions comprised cDNA synthesis 30 min at 50 ºC, denaturation for 2 min at 94ºC, followed by 40 cycles for 20 sec at 94ºC, 1 min at 60ºC, and 1 min at 68ºC, followed by an extension 68°C for 5 min.

3.4. Real time PCR of *A. phagocytophilum*

Samples were screened for the presence of *A.phagocytophilum* DNA by real-time PCR as described by Courtney (Courtney et al., 2004). Briefly, 5µl of each sample DNA was added to 20 µl of TaqMan Universal Master mix (Applied Biosystems, Branchburg, New Jersey USA), containing 0.9 µM of each primer

(ApMSP2f and ApMSP2r) and 200 mM of ApMS2-FAM probe. Cycling conditions comprised incubation 2 min at 50°C (to activate the enzyme AmpErase), 95°C for 10 min, followed by 45 cycles of a 15 sec denaturation step at 95°C followed by a 1 min annealing-extension step at 60°C. The 7900 Real Time PCR system (Applied Biosystems) was used for PCR reactions and fluorescent detections.

3.5. PCR amplification of partial *groESL*, 16S rRNA and *ankA* genes of *A. phagocytophilum*

Amplification of partial *groESL* operon was performed as described by Sumner (Sumner et al., 1997) and Lotric-Furlan (Lotric-Furlan et al., 1998). Briefly, partial *groESL* operon was amplified with forward primers HS1 (for the first PCR), HS43 (for nested PCR) and with reverse primers HS6 (for the first PCR) and HSVR (for nested PCR) (Table 1). PCR amplification was carried out in a total reaction volume of 25 µl, which contained the following mix of reagents: GeneAmp10xPCR buffer II (Applied Biosystems, Roche, Branchburg, NJ), $MgCl_2$ (1.5 mM), dNTPs (200 µM), 1 µM of forward and reverse primers for the first PCR or for nested PCR, AmpliTaq DNA polymerase (2.5 U) (Applied Biosystems, Roche, Branchburg, NJ), and 5 µl of target DNA. PCR conditions for the first step PCR consisted of a three cycles of 1 min at 94ºC, 2 min at 48°C and 1,5min at 70°C, followed by 37 cycles consisting of 1 min at 88ºC, 2min at 52°C and 1,5 min at 70°C, followed by an extension 68°C for 5min. PCR conditions for nested PCR consisted of a denaturation step of 2 min at 94ºC, followed by 30 cycles of 1min at 94°C, 2min at 55°C and 1,5min 72°C, followed by an extension 72°C for 10min.

Partial 16S rRNA gene was amplified with forward primers Ehr1 (for the first PCR), Ehr7 (for nested PCR) and with reverse primers Ehr6 (for the first PCR) and Ehr8 (for nested PCR) (Table 1) as described by Rar (Rar et al., 2005; Rar, 2010). PCR amplification was carried out in a total reaction volume of 25 µl, which contained the following mix of reagents: GeneAmp10xPCR buffer II (Applied Biosystems, Roche, Branchburg, NJ), $MgCl_2$ (2 mM), dNTPs (200 µM), 0.5 µM of forward and reverse primers for the first PCR or for the nested PCR, AmpliTaq DNA polymerase (2.5 U) (Applied Biosystems, Roche, Branchburg, NJ), and 5 µl of target DNA. PCR conditions for the fist PCR consisted of 35 cycles of 1min at 94°C, 1min at 63°C, 1,5 min at 72°C, followed by an extension step of 15 min at 72°C. PCR conditions for nested PCR were identical but annealing temperature was decrease up to 60°C.

The 3' portion of *ankA* gene was amplified as described by Massung and von Loewenich (Massung et al., 2000; von Loewenich et al., 2003) with forward primers 1F (for the first PCR), 2F1, 3F1 (for nested PCR) and with reverse primers 4R1 (for the first PCR), 4R1mod, 2R1 (for nested PCR) (Table 1). PCR amplification was carried out in a total reaction volume of 25 µl, which

contained the following mix of reagents: GeneAmp10xPCR buffer II (Applied Biosystems, Roche, Branchburg, NJ), $MgCl_2$ (2 mM), dNTPs (200 µM), 1 µM of forward and reverse primers for the first PCR or for the nested PCR, AmpliTaq DNA polymerase (2.5 U) (Applied Biosystems, Roche, Branchburg, NJ), and 5 µl DNA for the first PCR and 2 µl of PCR products for nested PCR. PCR conditions were 40 cycles for the fist PCR and 30 cycles for the nested PCR of 1min at 94°C, different annealing temperatures for 1min, 1,5 min at 72°C, followed by an extension step of 15 min at 72°C. Primers 1F and 4R1 were used for the first PCR with annealing temperature 55°C, product of this reaction was used for nested reactions with follow primers, 1F1 and 4R1mod at 55°C annealing temperature, 2F1 and 2R1 at 52°C, 3F1 and 4R1mod at 56°C.

3.6. PCR amplification of partial 18S rRNA gene of *Babesia* DNA and Reverse line blot hybridization

For screening of ticks, Reverse Line Blot (RLB) was performed as described earlier (Gubbels et al., 1999). Briefly, partial 18S rRNA gene of *Babesia* spp. was amplified with forward primer RLB-F2 and reverse primer RLB-R2 (Table 1). PCR amplification was carried out in total reaction volume of 50 µl, which contained the following mix of reagents: GeneAmp10xPCR buffer II, $MgCl_2$ (1 mM), dNTPs (200 µM), 0.5 µM of forward and reverse primers, AmpliTaq DNA polymerase (1.25 U) (Applied Biosystems, Roche, Branchburg, NJ), and 10 µl of target DNA. The PCR conditions consisted of a denaturation step of 3 min at 94ºC, followed by 10 cycles of touch down program, consisting of 20 sec of denaturation at 94ºC, and 30 sec of an annealing temperature decreased from 67ºC to 58ºC for each cycle, and an extension step of 30 sec at 72ºC, followed by 40 cycles of a denaturing step of 20 sec at 94ºC, an annealing step of 30 sec at 57ºC and an extension step of 30 sec at 72ºC. A final extension step of 10 min at 72ºC completed the program.

All the nucleotide sequences of the species-specific oligonucleotide probes containing a N-terminal N-(trifluoracetamidohexyl-cyanoethyle, N,N-diisopropyl phoshoramidite [TFA])-C6 amino linker, were synthesized by TAGC (Copenhagen, Denmark). The sequences of the oligonucleotide probes are shown in Table 1.

Preparation of RLB membrane, hybridization and subsequent stripping of the membrane were carried out as previously described (Gubbels et al., 1999), with the following modifications: the amplification reaction volume (50 µl) was loaded onto the blot after dilution with 2 x SSPE - 0.1% SDS to a total volume of 150 µl. The temperature of the two 10 min post-hybridisation washes in 2 x SSPE – 0.5 % SDS was increased to 51ºC. After developing, the PCR products were stripped from the membrane.

3.7. Sequencing of the partial 18S rRNA gene of *Babesia*

Samples positive by the RLB assay were amplified by nested PCR for sequencing of partial 18S rRNA gene with outer primers: PIRO-A and RLB-R2 and with inner primers: RLB-F2 and PIRO-B (Table 1). The PCR amplifications were performed in a total volume of 25 µl as follows: GeneAmp 10xPCR buffer II, $MgCl_2$ (1.5 mM) for the first PCR and (0.5 mM) for nested PCR, dNTPs for the first PCR (200 µM) and for nested PCR (400 µM), 1 µM of forward and reverse primers, AmpliTaq DNA polymerase (1.25 U) (Applied Biosystems, Roche, Branchburg, NJ), and 5µl of target DNA. The cycling conditions were an initial denaturation for 1 min at 94ºC, followed by 40 cycles for 1 min at 94ºC, 1 min at 59ºC, and 1 min at 72ºC. For the nested PCR the annealing temperature was 55ºC.

Table 1. Sequences of PCR primers and oligo probes used for the detection of TBEV, *A. phogocytophilium* and *Babesia spp*. in ticks.

Target organism/gene	**Names of primers/probes**	**Oligonucleotide sequence (5'→3')**	**References**
TBEV E long gene			
RT-PCR primer	41F1	TGGA(G/A)AACAGGGA(T/C)TTTGT(T/C/G)ACTGG	(Mickiene et al., 2001)
RT-PCR primer	1312R	T(G/A)AATGC(A/G)CCTCC(G/A)AG(G/A)ACTGT(G/A)TG	(Mickiene et al., 2001)
A. phagocytophilum			
Real-Time PCR primer	ApMSP2f	ATGGAAGGTAGTGTTGGTTATGGTATT	(Courtney et al., 2004)
Real-Time PCR primer	ApMSP2r	TTGGTCTTGAAGCGCTCGTA	(Courtney et al., 2004)
Real-Time PCR probe	ApMSP2-FAM	FAM-TGGTGCCAGGGTTGAGCTTGAGATTG-TAMRA	(Courtney et al., 2004)
***gro*ESL operon**			
I PCR primer	HS1	TGGGCTGGTA(A/C)TGAAAT	(Sumner et al., 1997)
I PCR primer	HS6	CCICCIGGIACIA(C/T)ACCTTC	(Sumner et al., 1997)
Nested PCR primer	HS43	AT(A/T)GC(A/T)AA(G/A)GAAGCATAGTC	(Sumner et al., 1997)
Nested PCR primer	HSVR	TCAACAGCAGCTCTAGTWG	(Liz et al., 2000)
16S rRNA gene			
I PCR primer	Ehr1	AACGAACGCTGGCGGCAAGC	(Rar et al., 2010b)
I PCR primer	Ehr6	GACCCAACCTTAAATGGCTGC	(Rar et al., 2010b)
Nested PCR primer	Ehr7	TAACACATGCAAGTCGAACG	(Rar et al., 2010b)
Nested PCR primer	Ehr8	CTTCGAGTTAAGCCAATTCC	(Rar et al., 2010b)
***Ank*A gene**			
I PCR primer	1F	ATGTTACGCTGTAATAGCATGGAC	(Massung et al., 2000)
I PCR primer	4R1	GCGACCTCCTTTTACAGACTTAG	(Massung et al., 2000)
Nested PCR primer	4R1mod	CTTTGAGGAGCTTCTGGTTG	(von Loewenich et al., 2003)

Nested PCR primer	2F1	CTGATGTAAATGCGTCTCCA	(Massung et al., 2000)
Nested PCR primer	2R1	ACCATTTGCTTCTTGAGGAG	(Massung et al., 2000)
Nested PCR primer	3F1	GTCTCGAAAGCATTTGTCAAAC	(Massung et al., 2000)
***Babesia* spp.**			
18S rRNA gene			
RLB probe	*Babesia* spp. („catch all“)	Amino-TAATGGTTAATAGGARCRGTTG	(Oura et al., 2004)
RLB probe	*B. microti*	Animo-CTTCCGAGCGTTTTTTTATT	(Garcia-Sanmartin et al., 2008)
RLB probe	*B. divergens*	Amino-GTTAATATTGACTAATGTCGA	(Gubbels et al., 1999)
PCR primer	RLB-F2	GACACAGGGAGGTAGTGACAAG	(Georges et al., 2001)
PCR primer	RLB-R2	biotin-CTAAGAATTTCACCTCTGACAGT	(Georges et al., 2001)
Nested PCR primer	PIRO-A	ATTACCCAATCCTGACACAGGG	(Armstrong et al., 1998)
Nested PCR primer	PIRO-B	TTAAATACGAATGCCCCCAAC	(Armstrong et al., 1998)

3.8. DNA sequencing

The amplified products of the nested PCR were purified with GFX^{TM}-PCR DNA (Amersham Biosciences, Sweden) and Gel Band purification kit (GE Healthcare UK Limited, UK) according to the manufacturer's instructions. The purified PCR products were used as templates in the sequencing reaction using the dideoxynucleotide chain termination method with BigDye Terminator v3.1 Cycle Sequencing Ready Reagent Kit (ABI PRISM, PE Applied Biosystems, California, USA). The ABI PRISM 3100 Genetic Analyser (Applied Biosystems) was used for sequence analysis. The obtained sequences were edited using the BioEdit program (www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned with the corresponding regions of sequences retrieved from GenBank.

3.9. Phylogenetic analysis

Phylogenetic analysis was carried out with the TREE-PUZZLE 5.2 version package. For phylogenetic tree reconstruction, a quartet puzzling Maximum Likelihood method was used and 10 000 for puzzling steps for *Anaplasma* and *Babesia* and 50 000 for puzzling steps for TBEV, respectively, were applied using the Hasegawa-Kishino-Yano (HKY) model for substitution (Hasegawa et al., 1985). The sequences were retrieved from the GenBank database and aligned by BioEdit program (www.mbio.ncsu.edu/BioEdit/bioedit.html).

4. RESULTS AND DISCUSSION

4.1. Genetic analyses of Siberian TBEV subtype in Estonia

4.1.1. Phylogenetic and amino acid analysis

A total of eight strains belonging to the TBEV-Sib subtype were isolated from *I. persulcatus* ticks in Estonia. Nucleotide sequences recovered from these strains included almost the complete E-gene (142-1247 nt). According to phylogenetic analysis (Fig. 6), the Estonian strains were clustered with the previously identified TBEV strains of the TBEV-Sib subtype isolated in Latvia, Estonia and Finland. These strains formed their distinct own Baltic lineage within the TBEV-Sib subtype. The second lineage within the TBEV-Sib subtype was formed by strains isolated in Siberia.

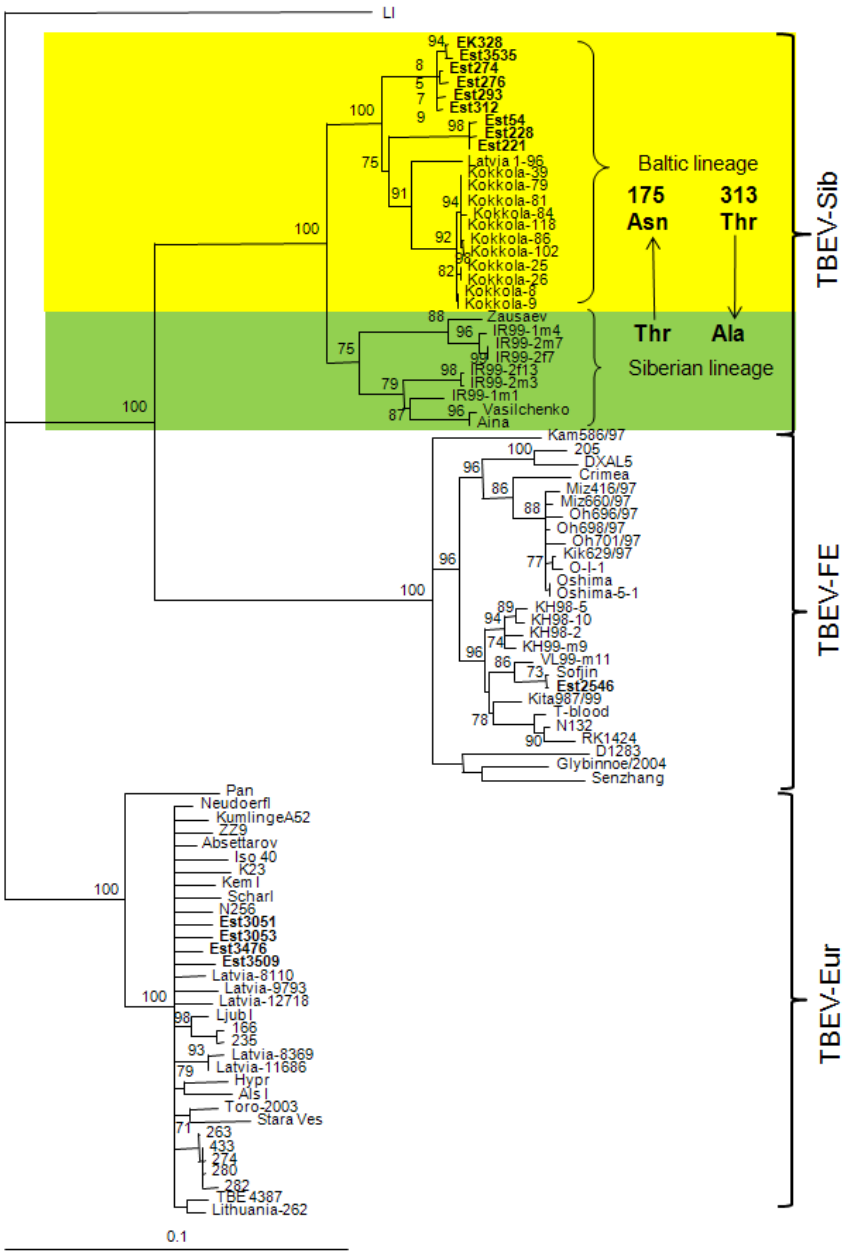

Figure 6. Phylogenetic tree (maximum likelihood) based on E glycoprotein encoding sequences (nt 142-1247). Bootstrap values > 70% (derived from 50 000 replicates of neighbour-joining trees estimated under ML substitution model) are shown.

Analysis of the deduced amino acid alignment of the partial E glycoprotein revealed in general the same signature amino acids for all subtypes as previously described (Ecker et al., 1999), as well as new signature amino acids within

TBEV-Sib. We found that the earlier published signature amino acids for the TBEV-Sib subtype (Ecker et al., 1999) at positions 119, 279, and 350 were not present in the majority of the TBEV-Sib strains. All Baltic strains of the TBEV-Sib subtype had a unique Thr/Asn substitution at position 175, previously not referred to as a signature amino acid. At position 313, all Siberian strains isolated from Novosibirsk, Tomsk and Irkutsk, of the TBEV-Sib subtype, had a unique Thr/Ala substitution and differed from Baltic strains of the same subtype and from strains belonging to the TBEV-FE and TBEV-Eur subtypes (Fig. 7).

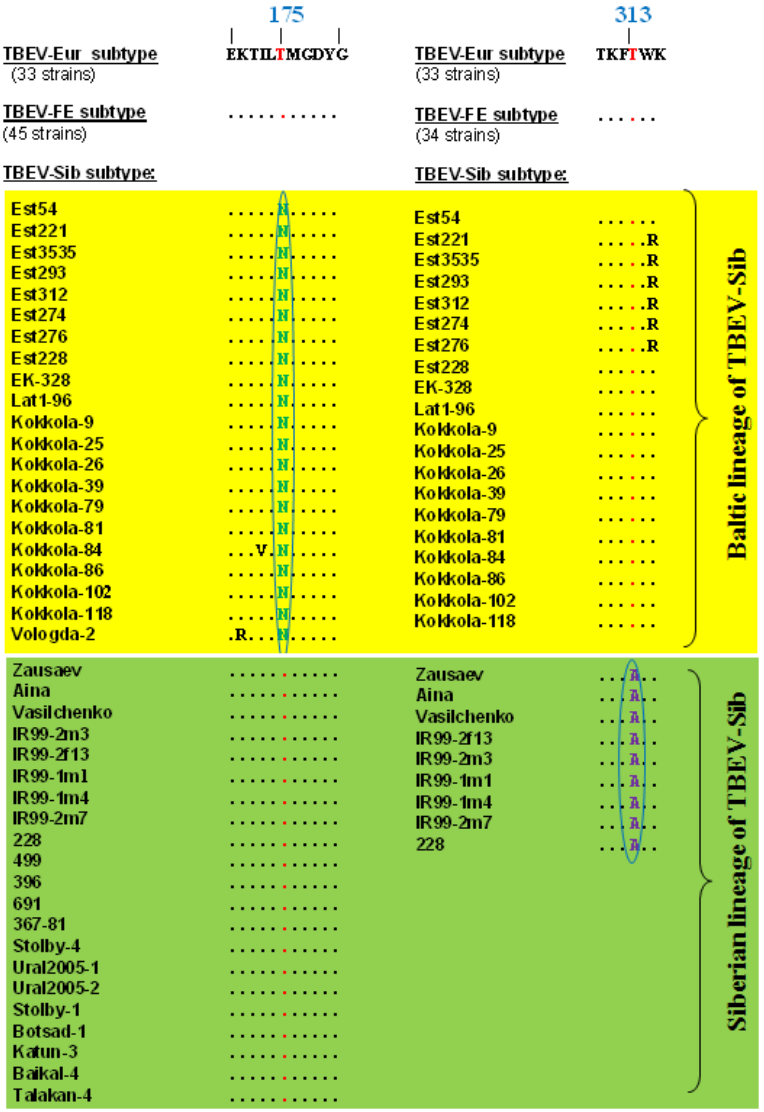

Figure 7. Comparison of signature amino acids of the E glycoprotein at positions 175 and 313 within the S-TBEV subtype. Amino acid alignment of the strain Neudoerfl is shown on top and dots indicate identical amino acids.

According to these findings, discrimination of Baltic and Siberian lineages within the TBEV-Sib subtype is possible. These data support the geographic clustering of Baltic TBEV strains within the TBEV-Sib subtype, in contrast to the previous postulation that TBEV strains could not be distinguished according to place and time of isolation.

4.1.2. Protein structure modeling

According to protein modeling and structure analysis of the protein E dimer molecule, it was shown that both signature amino acids 175 and 313 are located close to each other at the surface, at one side of the dimer molecule (Fig. 8a).

Geometrical analysis of the molecular surface around amino acid 313 suggested the presence of a cleft between this residue and a loop formed by residues 308-311, proposed as a putative flavivirus receptor binding site (Mandl et al., 2000) (Fig. 8b). Different hydrophobicity and polarity of Thr/Ala residues may influence the binding properties of the cleft formed by residue 313 and the 308-311 residue loop. E protein modeling suggested that at position 175, the differences in the polar properties of Thr and Asn could provide a structural difference between the E proteins of Siberian and Baltic lineages of TBEV-Sib. Strains belonging to the Siberian lineage within TBEV-Sib subtype have Thr at position 175, and this amino acid forms the hydrogen bond with Asp at position 181. Baltic strains have Asn at position 175, which does not form a hydrogen bond with Asp; a feature which makes loop 177 – 179 more flexible at the molecular surface of E protein (Fig. 8c).

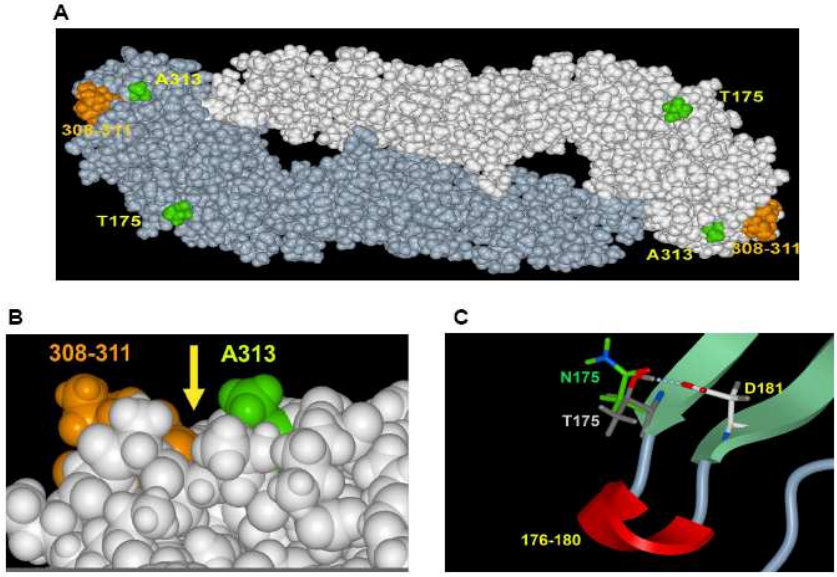

Figure 8. The E glycoprotein of the tick-borne encephalitis virus (TBEV). (a) Surface model. The two subunits of the dimer are coloured white and grey, respectively. The

amino acids 175 and 313 that distinguish the Baltic from Siberian lineages of the Siberian TBEV are coloured green and labelled A313 and T175. The putative receptor-binding site, the 308-311 loop, is coloured orange. (b) Model representation of signature amino acid Ala313 for Siberian lineage TBEV-Sib on the surface of the E glycoprotein. Atoms are shown in balls, Ala313 is coloured green and labelled A313, the 308-311 loop is coloured orange and labelled 308-311. The arrow indicates a cleft on the surface between residues 308-311 and 313. (c) Model representation of signature amino acid 175 for the Baltic lineages of TBEV-Sib strains on the surface of the E glycoprotein. The backbone is shown by ribbon representation; two β strand of antiparallel β sheet B in green, connected by labelled loop 176-180 containing the helical turn 177-179 and shown by red ribbon. The side chains of signature amino acid residues 175 are given in stick representation and coloured: green - Asn for Baltic lineage of TBEV-Sib (N175), and grey – Thr for the Siberian lineage (T175). Partly charged heavy atoms of residue polar groups are indicated with standard colours: oxygen with red and nitrogen with blue. Side chain of residue 181 is shown in stick representation and labelled as D181. The hydrogen bond between Asp181 and Thr175 in all TBEV subtypes, which is missing in the Baltic lineage of TBEV-Sib, is depicted.

The biological role of these findings is unclear, but the fact that both signature amino acids are situated on the viral surface close to the receptor-binding site, may constitute indirect evidence of the involvement of these signature amino acids in viral functional processes.

4.2. Genetic analysis of *Anaplasma phagocytophilum* in Estonia, the European part of Russia and Belarus

The objectives of the study were detection of *A. phagocytophilum* in ticks collected in Estonia, the European part of Russia and Belarus, and characterization by sequencing of the partial 16S rRNA gene, the *groESL* operon and the *ankA* gene.

A total of 3 976 ticks were collected in Estonia, among them 2 474 *I. ricinus* from mainland Estonia, 345 *I. ricinus* from Saaremaa island and 1 157 *I. persulcatus* from South-Eastern Estonia. In addition, ticks collected in neighbouring countries were analyzed: 477 ticks were collected in Russia, among them 82 *I. ricinus* from Curonian Spit and 395 *I. persulcatus* from the European part of Russia (St. Petersburg, Borok, Tcherepovets). In Belarus 187 of *I. ricinus* were collected in the Minsk area.

4.2.1. Prevalence of *A. phagocytophilum* in ticks

A. phagocytophilum was found only in *I. ricinus* ticks, in 1.7% of *I. ricinus* (42/2 474), collected at 8 out of 14 sites on mainland Estonia. On Saaremaa island *A. phagocytophilum* was detected in 9 out of 345 ticks with an overall prevalence of 2.6%. However, positive ticks were found only at 4 sites (Kamri,

Kotlandi, Rootsiküla, Üübide), where the prevalence varied from 6.3% to 12.5%. In Russia, 11 out of 82 (13.4%) *I. ricinus* collected on the Curonian Spit were found positive. In Belarus, *A. phagocytophilum* was detected in 8 out of 187 ticks (4.2%). In comparison with European countries the reported prevalence rates in *I. ricinus* ranging from 0.3 % in Western France (Cotte et al., 2009) to 17.1% on Norwegian islands (Rosef et al., 2009). As previously reported, ticks collected at different time points at the same locality had different rates of *A. phagocytophilum* infection (Jenkins et al., 2001). Furthermore, differences in prevalence may reflect seasonal or annual variation of tick infection rate, or different sampling, storage and methodical approaches.

According to our findings, *A. phagocytophilum* was found only in *I. ricinus*, despite that it has been detected previously in *I. persulcatus* in a neighbouring region of Russia (Alekseev et al., 2003). In Siberia and the Far East, *A. phagocytophilum* was detected in main vector *I. persulcatus* at a prevalence ranging from 1% to 9.6% (Ohashi et al., 2005; Rar et al., 2010b). In Estonia, even at two sites in south-eastern Estonia, where both species of ticks co-circulate and *I. ricinus* constitutes the minority (10-20%) of tick population, we found *A. phagocytophilum* only in *I. ricinus*. Therefore, the role of *I. persulcatus* in *A. phagocytophilum* circulation is unclear and repeated collection of ticks is necessary at sites where *I. persulcatus* is distributed.

4.2.2. Analysis of partial 16A rRNA gene

The partial 16S rRNA gene (1168 bp) was sequenced for fifteen samples found positive for *A. phagocytophilum* by real-time PCR. A high degree of similarity (98.9–100 %) of sequenced *A. phagocytophilum* strains has been found among the strains from Estonia, Belarus and Russia of which only five samples could be distinguished from each other while ten of them were identical. In order to distinguish the *A. phagocytophilum* variants we used a variable region near the 5'end of the 16S rRNA gene at position 76 to 84 (Cao et al., 2000; Chae et al., 2000; Massung et al., 2002; Stuen et al., 2002; Cao et al., 2003). Four genetic variants were found (Table 2).

Table 2. 16S rRNA gene variants detected in *I. ricinus* ticks collected in Estonia, European part of Russia and Belarus.

No of samples	Place of tick collection	16S variant	Nucleotide positions								
			76	77	78	79	80	81	82	83	**84**
Est 3229 Est 3238	Mainland of Estonia	1	A	A	A	G	A	A	T	A	G
Rus 30-10 Rus 30-13 Rus 34-7	Curonian Spit, Russia										
Bel 11-2-07 Bel 6-22-07 Bel-Bmi11	Minsk, Belarus										
Est2589	Estonia, Saaremaa	2	A	A	A	G	A	A	T	A	**A**
Est 1090	Mainland Estonia	3	**G**	A	A	G	A	A	T	A	**A**
Est 2477 Est 2535 Est 2540	Estonia, Saaremaa										
Rus 29-12	Curonian Spit, Russia										
Bel Bmi37	Minsk, Belarus	4	**G**	A	A	G	A	A	T	A	G

Variant 1 was revealed in eight samples from the Estonian mainland, the Russian part of Curonian Spit and Belarus; variant 2 was found in one sample from the Estonian island Saaremaa; variant 3 in five samples from mainland Estonia, Saaremaa island and the Curonian Spit (Russia); and variant 4 in only one sample from Belarus.

4.2.3. Analysis of *groESL* gene

A total of 1262 bp of the *groESL* heat shock operon were analyzed for the 21 samples found positive by the real-time PCR. The first 402 predicted amino acids of the groEL protein sequence were analyzed. Amino acid substitutions at position 139, (Glu→Arg in two Estonian strains) and at position 398 (Glu→Gly in one of the Belarusian strain) were found. According to the substitutions at amino acid position 242, all analyzed sequences (21 from this study and 38 retrieved from GenBank) divided the strains into two groups, one with serine (lineage 1) and another with alanine (lineage 2) at this position. We could confirm the splitting of *A. phagocytophilum* into two genetic lineages by phylogenetic analysis (Fig. 9).

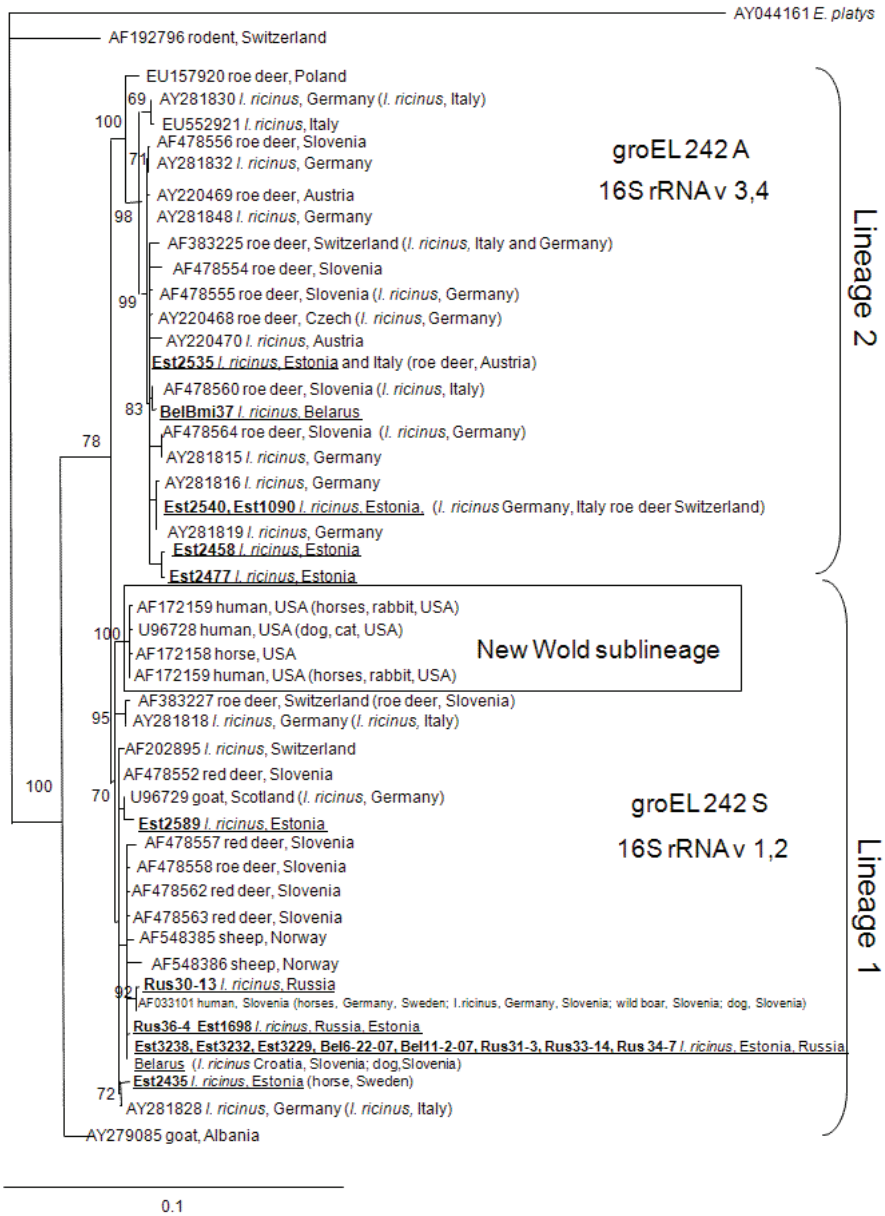

Figure 9. Phylogenetic tree (Maximum Likelihood) based on the partial sequence of *groESL* operon (nt 423 – 1679). Only bootstrap values > 70% (derived from 10 000 replicates neighbour-joining trees estimated under ML substitution model) are shown. Source and place of detection of identical sequences are shown in parentheses. Strains sequenced in our study are indicated in bold and underlined.

On the phylogenetic tree of the partial *groESL* operon sequences (1160bp) lineage 1 subdivided with high bootstrap support into the European and New World lineages, while lineage 2 included only European *A. phagocytophilum*

strains. Lineage 1 included strains of *A. phagocytophilum* pathogenic for humans or domestic animals (horses, dogs, sheep), while lineage 2 consisted of strains isolated from healthy animals as well as ticks. The strains analyzed in the present study belonged to both *A. phagocytophilum* genetic lineages; moreover, strains belonging to lineages 1 and 2 co-circulated at the same sites in Estonia, Belarus and in the Russian part of the Curonian Spit.

Strains of *A. phagocytophilum* belonging to 16S rRNA gene variants 1 and 2 grouped within lineage 1, while variants 3 and 4 grouped within *A. phagocytophilum* lineage 2 (Fig. 9).

4.2.4. Analysis of *ankA* gene

A total of eight samples were sequenced for the partial *ankA* gene and amplicons of different lengths were produced: 1591bp for the samples from Belarus (BelBmi11 and Bel6-22-07), 1642bp for the samples from Estonia and Russia (Bel11-2-07, Rus34-7, Rus30-13, Est3238), 1672bp for the Belarusian samples BelBmi37, and 1717bp for the Estonian sample Est3239. Sequencing revealed four distinct models of *ankA* gene organization (Fig. 10).

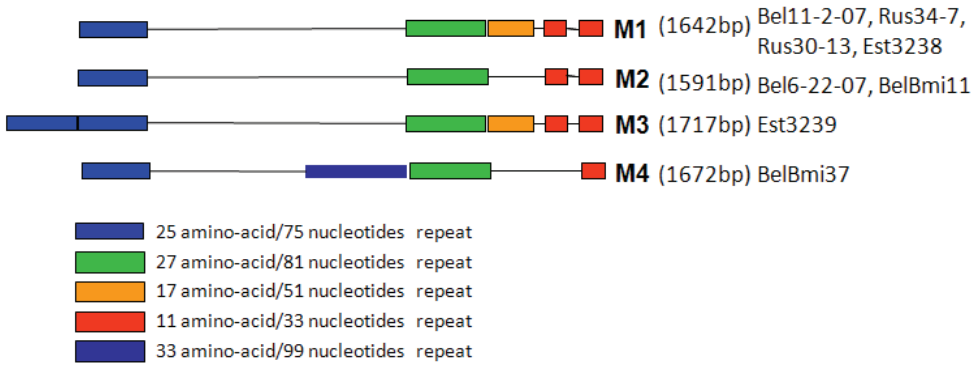

Figure 10. Schematic presentation of the variable numbers of repetitive elements within the *ankA* gene detected in this study. M1, M2, M3, M4 – *ankA* organization patterns 1, 2, 3 and 4.

In Bel11-2-07, Rus34-7, Rus30-13, Est3238 samples 25-, 27- and 17 amino-acid repeats respectively, were found as single copies and 11-amino-acid repeats in two copies.

Strains isolated from humans in Sweden and Slovenia have been shown to exhibit the same model of *ankA* gene organization (Massung et al., 2000), as has strains from horses (Germany, Switzerland), dogs (Germany, Denmark), and sheep (Germany) (von Loewenich, unpublished, GenBank).

One copy of 25- and 27-amino-acid repeats, no 17- amino-acid element, and two copies of 11- amino-acid repeats were found for Bel6-22-07 and BelBmi11 samples. This pattern has previously been demonstrated only in one sample from a dog in Austria (von Loewenich, unpublished, GenBank).

One sequence of an Estonian sample (Est3239) included two copies of a 25-amino-acid repeat, one copy of 27- and 17-amino-acid elements and two copies of an 11-amino-acid repeat *A. phagocytophilum* strains from ticks and dogs from Germany (von Loewenich et al., 2003), as well as from Switzerland (von Loewenich, unpublished, GenBank), have been shown to have a similar pattern of organization.

Sample BelBmi37 included one copy of 25-, 27-and 11-amino-acid elements, and a 33-amino-acid insertion located directly adjacent to, and upstream from a 27-amino-acid repeat. Strains from *I. ricinus* collected in Germany (von Loewenich et al., 2003) and samples from roe deer in Germany, Slovenia, Spain and Norway (von Loewenich, unpublished, GenBank) have been revealed to posess a similar model of ankA organization.

We found that the strains of *A phagocytophilum* with different patterns of *ankA* organization may co-circulate in the same geographical region, e.g. sequences of *ankA* gene belonging to models 1 and 3 were detected at the same sites in Estonia, while models 1, 2 and 4 were found in Belarus.

According to phylogenetic analysis of partial *ankA* gene the strains of *A. phagocytophilum* formed two lineages (Fig. 11), and the distinction between lineages 1 and 2 corresponded well to the lineage splitting based on the *groESL* phylogenetic tree.

A. phagocytophilum within lineage 1 and sequenced for the *groESL, ankA,* and 16S rRNA genes were classified as 16S rRNA gene variants 1 and 2, while variants 3 and 4 were detected within lineage 2.

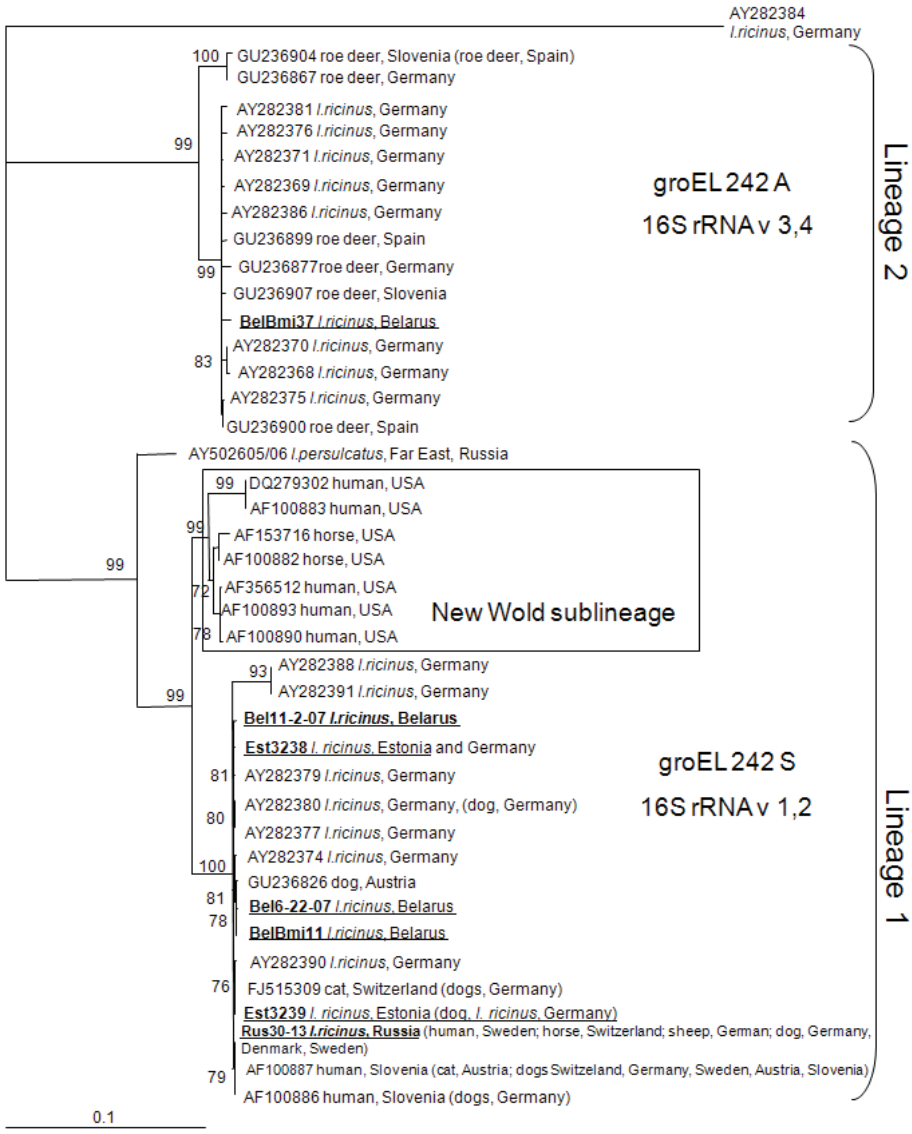

Figure 11. Phylogenetic tree (Maximum Likelihood) based on the partial sequence of *ankA*. Only bootstrap values > 70% (derived from 10 000 replicates neighbour-joining trees estimated under ML substitution model) are shown. Source and place of detection of identical sequences are shown in parentheses. Strains sequenced in this study are indicated in bold and underlined.

The present study demonstrated that *A. phagocytophilum* strains belonging to different *groESL* lineages, with different variants of 16S rRNA genes and with different patterns of *ankA* gene organization, co-circulated in the same geographical regions in Estonia, Russia and Belarus. We suggest that ticks may harbour all repertoires of *A. phagocytophilum* strains that are circulating in this

region, while genetic variants of *A. phagocytophilum* segregate in specific natural hosts. Thereby ticks may be an optimal source for studies on the diversity of *A. phagocytophilum* in various geographical regions.

4.3. Genetic analyses of *Babesia* spp. in ticks in Estonia

The present study revealed for the first time the presence of *Babesia* in Estonia. A total of 2603 ticks including 938 *I. persulcatus* (577 adults and 361 nymphs) and 1665 *I. ricinus* (587 adults and 1078 nymphs) were collected from different parts of Estonia during 2006-2008 (Fig. 12).

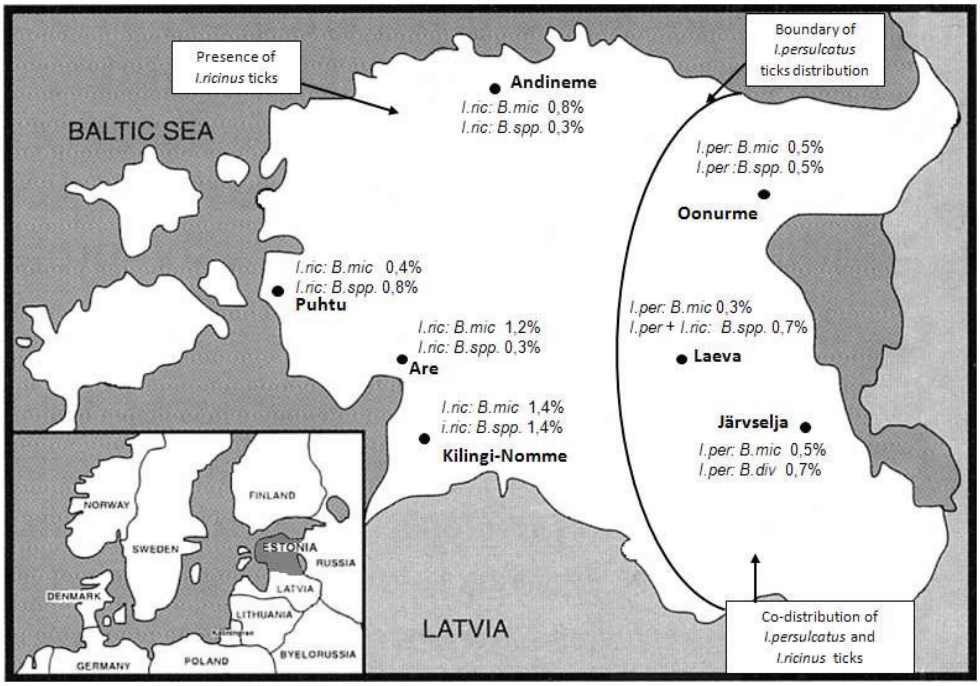

Figure 12. Tick sampling sites in Estonia (Andineeme, Oonurme, Laeva, Järvselja, Kilingi-Nõmme, Are, Puhtu) and the prevalence of *Babesia* spp. The areas of *I. persulcatus* and I. *ricinus* distribution are indicated.

All ticks were tested for the presence of *Babesia* spp. by reverse line blot (RLB) hybridization. The presence of *Babesia* was detected in 36 pools of ticks (1.4%); among them 18 (0.7%) were recognized by the *B. microti* probe, three (0.1%) by the *B. divergens* probe, and the other 15 (0.6%) were recognized only by the universal *Babesia* spp. "catch all" probe on the membrane (Table 3). *B. microti* was detected in four pools of *I. persulcatus* (3 pools of females and 1 pool of males) from the eastern part of Estonia (Laeva, Oonurme and Järvselja)

and in 14 *I. ricinus* nymph pools, which were collected in the western part of Estonia (Andineeme, Puhtu, Are and Kilingi-Nõmme). Three *I. persulcatus*-pools (2 males and 1 female) from the Järvselja collection site reacted with the *B. divergens* probe. Fifteen tick pools (13 pools of *I. ricinus* nymphs and 2 of *I. persulcatus*) with undetermined *Babesia* species by RLB, originated from Oonurme, Puhtu, Laeva, Andineeme, Kilingi-Nõmme and Are. The minimum infection rate (MIR) was calculated by assuming that only 1 tick out of 5 in a pool was positive. The prevalence rates of *Babesia* species at different geographical sites are shown in Figure 12 and Table 3.

Table 3. *Babesia* spp. detection in ticks by reverse line blot hybridization.

Sampling sites	Tick species	*B. microti* No. of infected samples/Nr. of investigated samples (MIR %)*	*B. divergens* No. of infected samples/Nr. of investigated samples (MIR %)*	*Babesia* spp. No. of infected samples/Nr. of investigated samples (MIR %)*	Total No. of infected samples/Nr. of investigated samples (MIR %)*
Puhtu	*I. ricinus*	2/475 (0.4%)	0/475	4/475 (0.8%) (2 - EU1) †	6/475 (1.3%)
Are		4/321 (1.2%)	0/321	1/321 (0.3%)	5/321 (1.5%)
Andineme		3/359 (0.8%)	0/359	1/359 (0.3%) (1 - EU1) †	4/359 (1.1%)
Kilingi-Nõmme		5/345 (1.4%)	0/345	5/345 (1.4%) (1 - EU1) †	10/345 (2.9%)
Oonurme	*I. persulcatus*	1/209 (0.5%)	0/209	1/209 (0.5%)	2/240 (0.8%)
	I. ricinus	0/31	0/31	0/31	
Laeva	*I. persulcatus*	1/320 (0.3%)	0/320	1/320 (0.3%)	4/417 (0.9%)
	I. ricinus	0/97	0/97	2/97 (2%) (2 - EU1) †	
Järvselja	*I. persulcatus*	2/409 (0.5%)	3/409 (0.7%)	0/409	5/446 (1.1%)
	I. ricinus	0/37	0/37	0/37	
Total		18/2603 (0.7%)	3/2603 (0.1%)	15/2603 (0.6%) (6 - EU1) †	36/2603(1.4%)

* MIR – minimum infection rate

† No of samples confirmed as EU1 by sequencing

4.3.1. Analysis of partial 18S rRNA gene

For confirmation of the Babesia findings, 16 samples found positive by RLB (8 recognized by the *B. microti* probe, 2 recognized by the *B. divergens* probe, and 6 recognized by the *Babesia* spp. "catch all" probe) were amplified and sequenced in the 18S rRNA gene region.

B. mictoti DNA was amplified from *I. persulcatus* (Est603, Est884) and *I. ricinus* (Est 923, Est939, Est941, Est1033, Est1078, Est1098). Seven out of eight amplicons were found identical to the 378 bp amplicon of the 18S rRNA gene sequences previously found in a human blood sample (GenBank EF413181) in Germany, and also to sequences from *I. ricinus* ticks and rodents from Slovenia (GenBank AF373332, AY149572) and Switzerland (GenBank AF494286). However, the sequence of the *B. microti* sample Est884 differed from the other Estonian sequences by two nucleotide substitutions at positions 614 (A/G) and 699 (T/C), which made it distinct from other published sequences by at least 1 substitution. On the phylogenetic tree, the Estonian *B. microti* samples clustered together with strains belonging to the zoonotic "US"-type, which is distributed worldwide, and has been reported as pathogenic for humans (Grey et al., 2010) (Figure 2). The Estonian sequences share a high rate of similarity (99.7 – 100%) with other sequences belonging to the "US"-type; 98.4% with sequences belonging to the Hobetsu- and Kobe-types, while the lowest similarity rate (95.8-96.3%) was demonstrated with Munich- type strains. *B. microti* was the most frequently found *Babesia* species in Estonia. We found identical *B. microti* sequences in *I. persulcatus* and *I. ricinus* ticks; thus we suggest that both tick species play a role in transmission and maintenance of *B. microti* in Estonian natural foci.

The two *B. divergens*-like samples (Est622, Est746) were amplified from *I. persulcatus* ticks and generated 350 bp amplicons of the partial 18S rRNA gene. They were identical to sequences previously detected in roe deer and reported as *B. divergens* in Slovenia (GenBank AY572456), Poland (GenBank DQ083544) and Spain (GenBank DQ866844), while in France identical sequences were classified as *B. capreoli* (GenBank AY726009, FJ944827, FJ944828). Therefore, the distinction between the two closely related *Babesia* species, *B. divergens* and *B. capreoli* remains questionable. We reported our Estonian sequences as *B. divergens*-like, although the Estonian samples had G at nucleotide position 631 and T at nucleotide position 663 of the 18S rRNA gene, as that was proposed as feature of *B. capreoli* (Malandrin et al., 2010). Other *B. divergens* and *B. divergens*-like sequences from Europe, USA and Western Siberia showed 98.2 – 99.7% identity with the Estonian samples amplified in the present study. We found *B. divergens*-like (probably *B. capreoli*) sequences only in *I. persulcatus* ticks, while in other European countries (Slovenia, Germany, and Switzerland) this species of *Babesia* was detected in *I. ricinus* (Duh et al., 2001; Hartelt et al., 2004; Casati et al., 2006). Moreover, the Estonian strains

were identical to the other European strains in the partial 18S rRNA gene region; thus we suggest that *B. divergens*-like strains could use both tick vectors for transmission and maintenance in natural foci (Fig. 13).

The six sequenced samples (Est788, Est670, Est916, Est1148, Est1362, and Est1444) out of the 15 ones found positive by RLB only with the universal *Babesia* spp. probe were identified as EU1 after sequencing. All six were amplified from *I. ricinus* ticks and produced 350 bp amplicons that were identical to each other and to the *Babesia* sp. EU1 sequences detected from *I. ricinus* in France (GenBank FJ215873) and Slovenia (GenBank AY553915), from roe deer in Slovenia (GenBank AY572457) and France (GenBank EF185818), from human blood in Italy (GenBank AY046575), and from *I. persulcatus* in Western Siberia (GenBank GU734773) (Fig. 13). In the present study, *Babesia* sp. EU1 was detected only in *I. ricinus* ticks even in areas where *I. persulcatus* predominated (85% of collected ticks). Recently it was demonstrated that *I. ricinus* is a competent vector for *Babesia* sp. EU1 (Bonnet et al., 2007; Becker et al., 2009). However, *Babesia* sp. EU1 was found also in *I. persulcatus,* the suggested vector in Western Siberia (Rar et al., 2010a). Our Estonian sequences were identical to the European as well as Siberian ones in the partial 18S rRNA gene region (Fig. 13). Thus it needs to be clarified if *Babesia* sp. EU1 is not able to utilise *I. persulcatus* as a vector in Estonia, or if the detection of this *Babesia* species only in *I. ricinus* is due to a collection bias.

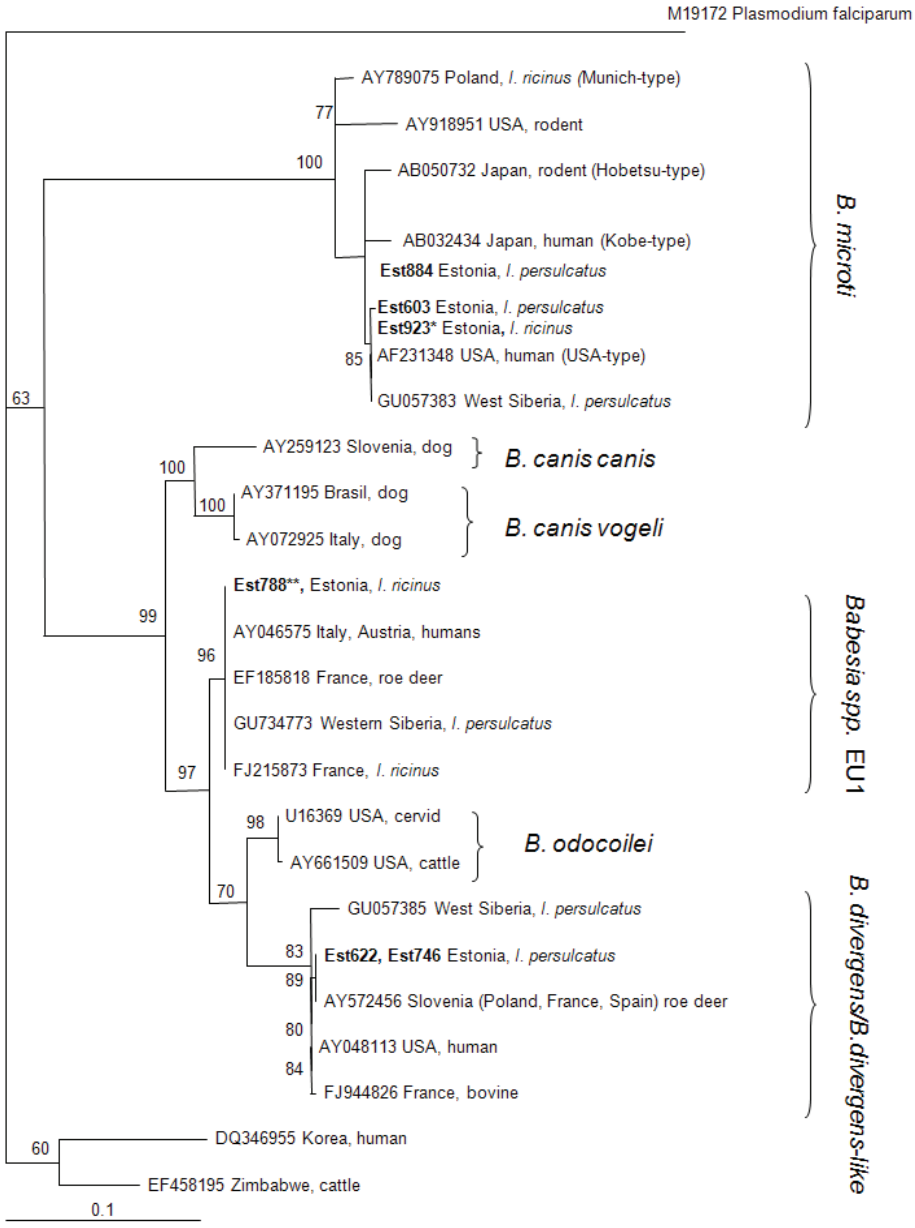

Figure 13. Phylogenetic tree based on partial 18S rRNA gene (378 bp) sequences of *Babesia* species. The phylogenetic tree was constructed by using the quartet puzzling maximum likelihood method (TREE-PUZZLE program). The numbers on the nodes are bootstrap values based on 10 000 replicates (only values greater than 70% are shown). *Plasmodium falciparum* has been used as the outgroup. The GenBank accession numbers for the sequences used in the present study are given. ***Est923** is identical to sequences **Est939, Est941, Est1033, Est1078, Est1098.** ****Est788** is identical to sequences **Est670, Est916, Est1148, Est1362, Est1444.**

On the phylogenetic tree based on the partial 18S rRNA gene, the Estonian sequences clustered together with other *B. microti*, *B. divergens/B. divergens-like* and *Babesia* sp. EU1 sequences from Europe, USA and Western Siberia with a high bootstrap support. Moreover, the analyzed sequences of *B. microti* and *Babesia* sp. EU1 were identical in the 18S partial gene region to sequences reported in symptomatic humans. To date there have, however, been no reports of human infections due to *Babesia* spp. in Estonia.

The results of the present study demonstrated for the first time the presence *B. microti*, *B. divergens*-like and *Babesia* sp. EU1 in natural populations of *Ixodes* ticks in Estonia, which should be considered as a possible risk of transmission to humans.

CONCLUSIONS

1. Phylogenetic analyses of the Estonian TBEV strains, isolated from *I.persulcatus* ticks, showed that they clustered together with strains isolated in Latvia and Finland. The strains formed their own lineage within the Siberian subtype, which we named Baltic lineage. Analysis of predicted amino acid sequences revealed new signature amino acid substitutions at position 175 for the Baltic lineage and at position 313 for the Siberian strains within the TBEV-Sib subtype. E protein structure modeling demonstrated that both signature amino acids are located close to each other and to the putative receptor-binding site at one side of the E protein dimer molecule and it was suggested, that they could be involved in cell receptor binding.

2. The presence of *A. phagocytophilum* was detected in *I. ricinus,* but not in *I. persulcatus* ticks, from different regions of Estonia. The genetic analysis of *A. phagocytophilum* sequences revealed different variants of *groELS*, 16S rRNA and *ankA* genes of local Estonian strains. A phylogenetic analysis of the *groESL* and *ankA* genes showed that the *A. phagocytophilum* strains divided into two lineages, consisting of pathogenic and non-pathogenic strains, respectively. The Estonian strains belonged to both *groESL* and *ankA* lineages.

3. For the first time, the presence of three *Babesia* species, *B. divergens*, *B. microti* and *Babesia* sp. EU1, were detected in ticks from different regions of Estonia. Genetic characterization of the Estonian sequences, based on partial 18S rRNA gene, showed that the Estonian strains were closely related to strains circulating in other European countries, Siberia and USA.

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ABSTRACT

Tick-borne pathogens (TBP) are widely distributed, and play an important role in human morbidity all over the world. Tick-borne diseases (TBD) are infectious zoonotic diseases occurring in endemic areas or natural foci where the circulation of TBP is maintained over long periods of time. Tick-borne encephalitis virus (TBEV) is a well studied TPB and the disease caused by it, tick-borne encephalitis (TBE), is widely distributed and well recognized in Europe and in Estonia. There is also an increasing number of cases of human granulocytic anaplasmosis (HGA) and human babesiosis reported in Europe; diseases caused by *Anaplasma phagocytophilum* and *Babesia* species, respectively. The *Ixodes* ticks are the main vectors of TBP in Europe. There are two tick species of the *Ixodes* genus circulating in Estonia: *I. ricinus,* prevalent throughout the entire territory, and *I. persulcatus,* found in Eastern Estonia. The co-circulation of two *Ixodes* species is unique for Europe, where only *I. ricinus* circulates, and therefore studies of the presence, circulation and genetic characteristics of TBP in Estonia are important and significant.

In the present study, an analysis of specific amino acid substitutions in the E glycoprotein of the Estonian TBEV strains belonging to the Siberian subtype was carried out. A study of the circulation as well as genetic characterization of the zoonotic tick-borne pathogens *Anaplasma phagocytophilum* and *Babesia* species *B. microti*, *B. divergens*-like and *Babesia* sp. EU1, were performed in Estonia for the first time.

The first goal of this thesis was to analyze strains of the TBEV- Siberian subtype circulating in Estonia. Phylogenetic analyses showed that Estonian strains and strains from Latvia and Finland form a well supported Baltic lineage within the TBEV-Sib subtype. We found a new unique signature amino acid substitution within the Siberian subtype of TBEV at position 175 for Baltic strains and 313 for Siberian strains, and could thus discriminate the Baltic TBEV-Sib strains from strains isolated in Siberia and the Far-East. Furthermore, protein structure modelling and geometrical analysis of the E protein surface showed, that both amino acids are located close to each other and to the receptor-binding site and may influence the receptor binding properties of the virus.

The second goal of this study was to investigate the presence and prevalence of *A. phagocytophilum* in Estonian ticks and to provide a genetic characterization and phylogenetic analysis of local *A. phagocytophilum* strains. The present study showed that *A. phagocytophilum* was detected only in *I. ricinus* ticks from all investigated sites with prevalence rates varying from 1.7 to 2.6%. Genetic analysis demonstrated that the Estonian strains of *A. phagocytophilum* belonged to different *groESL* lineages, and represented different variants of 16S rRNA and the *ankA* genes. We suggest that ticks may harbor all repertoires of *A. phagocytophilum* that are circulating in the same area, while genetic variants of *A. phagocytophilum* segregate in specific natural

hosts. Thereby ticks may be an optimal source for studies on the diversity of *A. phagocytophilum* in various geographical regions.

The last goal of this thesis was to analyze *Babesia* species circulating in Estonia. In the present study, the circulation of three Babesia species, *B. microti*, *B. divergens*-like and *Babesia* sp. EU1, was detected, with prevalence rates of up to 1.4% in Estonian ticks. *B. microti* was detected in both tick species, while *Babesia* sp. EU1 and *B. divergens*-like were only found in *I. ricinus* and *I. persulcatus*, respectively. Genetic studies based on the analysis of partial 18S rRNA gene revealed that Estonian sequences of *Babesia* species shared a high rate of similarity and were closely related to *B. microti*, *B. divergens*-like and *Babesia* sp. EU1 strains from other European countries, Siberia and the USA. Moreover, the analyzed sequences in the 18S partial gene region of *B. microti*, *B. divergens*-like and *Babesia* sp. EU1 were identical to sequences reported in infected patients; however, human infections due to *Babesia* species have to date not been reported in Estonia.

KOKKUVÕTE

Puukidega ülekantavad haigustekitajad on laialt levinud ja neil on oluline roll inimeste haigestumises kogu maailmas. Puugid vahendavad nakkuslike zoonootilisi haigusi, mis levivad endeemilistel aladel ja looduslikes kolletes, kus nad püsivad pikka aega. Puukentsefaliidiviirus (PEV) on puukidega ülekantav haigustekitaja ning selle põhjustatud haigus puukentsefaliit on laialt levinud ja hästi uuritud nii Euroopas kui ka Eestis. Üha sagenevad ka inimeste granulotsütaarse anaplasmoosi ja inimeste babesioosi juhtumid Euroopas. Nende haiguste põhjustajaiks on vastavalt bakter *Anaplasma phagocytophilum* ja algloom *Babesia* liigid. Perekonnast *Ixodes* pärit puugid on peamised vektorid puukidega ülekantavate haigustekitajate jaoks Euroopas. Eestis ringlevad kaks puugiliiki perekonnast *Ixodes*: *I. ricinus*, mis on levinud kogu territooriumil, ja *I. persulcatus*, mida on leitud Ida-Eestis. Selline erinevate puugiliikide levikualade kattumine on Euroopa jaoks, kus ringleb ainult *I. ricinus,* unikaalne, seetõttu on uuringud puukidega ülekantavate haigustekitajate olemasolust, nende tsirkulatsioonist ja geneetilisest iseloomustustest Eestis väga tähtsad ja märkimisväärsed.

Käesolevas töös teostati uue unikaalse tunnusaminohappe asenduse analüüs E- valgul Eesti PEV tüvedel, mis kuuluvad Siberi alltüüpi. Ühtlasi tehti uuringud haigustekitajate olemasolu ja tsirkulatsiooni osas ning anti esmakordselt Eestis geneetiline iseloomustus liikidele *Anaplasma phagocytophilum* ja *Babesia* (*B. microti*, *B. divergens*-laadne, *Babesia* sp. EU1).

Käesoleva töö üheks eesmärgiks oli analüüsida Eestis tsirkuleerivaid PEV Siberi alltüübi tüvesid. Fülogeneetiline analüüs näitas, et Eesti tüved ning Lätis ja Soomes isoleeritud tüved moodustavad hästi kindlustunud Balti liini PEV Siberi alltüübi sees. Leiti uue unikaalse tunnusaminohappe asendus E-valgul PEV Siberi alltüübi sees, positsioonis 175 Balti tüvedes ja positsioonis 313 Siberi tüvedes, mis võimaldab eristada Balti tüvesid Kaug-Idas ja Siberis levivaist. Lisaks näitasid valkude struktuuri modelleerimine ja pinnavalgu E geomeetriline analüüs, et mõned aminohapped asuvad lähestikku retseptorseondumise piirkonnaga, mis omakorda võib mõjutada viiruse retseptorsiduvaid omadusi.

Teiseks eesmärgiks oli uurida haigustekitaja *A. phagocytophilum* olemasolu ja levikut Eesti puugipopulatsioonis ja teha Eestis levinud *A. phagocytophilum*i tüvede geneetiline ja fülogeneetiline analüüs. *A. phagocytophilum* leiti ainult puugiliigis *I. ricinus* erinevatel aladel esinemusega 1,7–2,6%. Geneetiline analüüs näitas, et *A. phagocytophilum* Eesti tüved kuuluvad erinevatesse *groESL* liinidesse ning et esindatud olid erinevad 16S rRNA ja *ankA* geenide variandid. Arvame, et puugid võivad olla kogu *A. phagocytophilum*i repertuaari vektoriteks, mis ringlevad samas piirkonnas, samal ajal *A. phagocytophilum*i geneetilised variandid eraldatakse looduslike peremeeste vahel. Seejuures võivad puugid olla optimaalseks allikaks *A. phagocytophilum*i mitmekesisuse uuringutes eri geograafilistes piirkondades.

Kolmas eesmärk oli uurida *Babesia* liigi olemasolu ja levikut Eestis. Leiti kolm *Babesia* liiki – *B. microti, B. divergens* ja *Babesia* EU1 – erinevatel aladel levimusega 1,4% puugipopulatsioonist. *B. microti* leidus mõlemas puugiliigis, *Babesia* EU1 ainult liigis *I. ricinus* ja *B. divergens*-laadne liigis *I. persulcatus*. Geneetilisel analüüsil, mis tehti osalise 18S rRNA geeni põhjal, saime teada, et Eestis levinud *Babesia* liigi järjestused on kõrge sarnasusega seotud *B. microti*, *B. divergens*-laadse ja *Babesia* EU1 tüvedega teistest Euroopa maadest, Siberist ning USAst. Ka olid analüüsitud *B. microti*, *B. divergens*-laadse ja *Babesia* EU1 järjestused osalise 18S rRNA geeni piirkonnas identsed järjestustega haigestunud inimestelt. *Babesia* liiki patogeeni põhjustatud haigusjuhte pole Eestis veel registreeritud.

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PUBLICATION I

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Unique signature amino acid substitution in Baltic tick-borne encephalitis virus (TBEV) strains within the Siberian TBEV subtype

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Abstract

Tick-borne encephalitis virus (TBEV) is an arthropod-borne virus, which is transmitted to vertebrates by the bite of infected ticks. TBEV plays an important role in human morbidity in Europe and in Estonia in particular. All three known TBEV subtypes, Western (W-TBEV), Far-Eastern (FE-TBEV), and Siberian (S-TBEV), co-circulate in Estonia. In the present study, we collected ticks in the eastern part of the country where one of the TBEV vectors, *Ixodes persulcatus*, is prevalent. In total, 8 TBEV strains were isolated and characterized by partial sequencing of the surface E glycoprotein gene. Phylogenetic analysis showed that all 8 strains belonged to the S-TBEV subtype and clustered geographically with Baltic TBEV strains from Estonia, Latvia, and Finland. Analysis of amino acid sequences revealed a new signature amino acid, Asn, at position 175 for Baltic strains from Estonia, Latvia, Finland, and the European part of Russia, and Ala at position 313 for Siberian strains from Novosibirsk, Tomsk, and Irkutsk within the S-TBEV subtype. According to these findings, discrimination of Baltic and Siberian lineages within the S-TBEV subtype is possible. These data support geographic clustering of Baltic TBEV strains within the S-TBEV subtype in contrast to the previous postulation that TBEV strains could not be distinguished according to place and time of isolation. Both signature amino acids, 175 and 313, are located close to each other at one side of the E protein dimer molecule. Protein structure modeling showed that at position 175, the Baltic strains of S-TBEV had lost one hydrogen bond with Asp181, thus making the nearby 177–179 loop more flexible at the molecule surface. At position 313, the Siberian strains of S-TBEV had a substitution of non-polar Thr to polar Ala. Geometrical analysis of the molecular surface around amino acid 313 hinted at the presence of a cleft between this residue and a loop formed by residues 308–311, which has been suggested as a putative flavivirus receptor-binding site. This substitution may influence the binding properties of the cleft formed by signature amino acid 313 and the receptor-binding loop.

Keywords: Tick-borne encephalitis virus (TBEV); Signature amino acids; Siberian subtype of TBEV (S-TBEV); E glycoprotein; *Ixodes* ticks; Phylogenetic relationships

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Introduction

Tick-borne encephalitis virus (TBEV) belongs to the tick-borne flavivirus group, genus Flavivirus, family Flaviviridae. The TBEV genome consists of a single positive strand of RNA, approximately 11 kb, which contains one open reading frame (ORF) and encodes for a large polyprotein, which is cleaved into 10 proteins. Two proteins are located on the viral surface, the membrane protein (M) and the envelope glycoprotein (E). The E glycoprotein is responsible for the essential functions during the flavivirus life cycle, including host cell receptor binding (Rice, 1996) and thus determines, at least in part, the cell tropism and pathogenesis of the virus. The E glycoprotein is also responsible for inducing neutralizing antibodies and protective immunity (Heinz et al., 1990). The atomic structure of the soluble dimeric fragment of the E glycoprotein has been shown at a resolution of 2.0 Å (Rey et al., 1995). Two monomeric subunits form a head-to-tail dimer, and each of the monomers is composed of three domains: I, which forms a β barrel; II, which projects along the virus surface between the transmembrane regions of the homodimer subunits; and III, which maintains an immunoglobulin constant region-like fold, thought to be the receptor-binding region (Rey et al., 1995; Mandl et al., 2000). Binding sites for antibodies have been mapped to different locations on the entire surface of each of the three domains, which suggested that all three domains were antigenically active (Mandl et al., 1989).

TBEV causes TBE in humans after the bite of an infected tick. Two different types of hosts are needed for the survival of TBEV: (i) ticks that act as virus reservoirs and vectors and (ii) vertebrate hosts, which act as a source of blood for feeding ticks and support TBEV transmission by co-feeding of infected and non-infected ticks on the same host (Labuda et al., 1993). Two tick species, *Ixodes ricinus* and *I. persulcatus*, are the main vectors of TBEV. The geographical distribution of *I. ricinus* includes large areas of Europe and parts of North Africa. *I. persulcatus* has a vast range in Eurasia, from the Baltic countries Estonia and Latvia in the west to northern Japan in the east. Natural TBE foci coincide with the distribution of these vector ticks.

Based on phylogenetic analysis, three subtypes of TBEV have been described, the Western (W-TBEV) subtype transmitted by *I. ricinus*, the Far-Eastern (FE-TBEV), and the Siberian (S-TBEV) subtypes transmitted by *I. persulcatus* (Ecker et al., 1999). Amino acid substitutions, called signature amino acids, specific for each TBEV subtype have been identified on the E protein (Ecker et al., 1999) and on the basis of these markers TBEV subtypes are easily distinguished from each other.

The W-TBEV subtype is widely distributed across Europe with strains having been isolated in Austria, Switzerland, Sweden, Germany, Hungary, Czech Republic, Slovenia, Finland, Latvia, Lithuania, Estonia, Belarus, and the European part of Russia (Ecker et al., 1999; Lundkvist et al., 2001; Haglund et al., 2003). FE-TBEV and S-TBEV strains are spread from Japan and Far East Russia to the Baltic-Nordic countries, Latvia, Estonia, and Finland (Lundkvist et al., 2001; Mickiene et al., 2001; Jaaskelainen et al., 2005).

In our previous study, we found that within the S-TBEV subtype, three Estonian and one Latvian TBEV strains had unique signature amino acids and that this substitution may discriminate TBEV strains isolated in the Baltic countries and Finland from strains isolated in Siberia. At position 175, Baltic and Finnish strains and at position 313 Siberian strains were distinguishable, not only from each other within the S-TBEV subtype but also from all strains belonging to the W- and FE-TBEV subtypes (Golovljova et al., 2004). In order to confirm the hypothesis that Baltic and Siberian S-TBEV strains acquire unique signature amino acids, which could be regarded as genetic markers for these strains according to a geographical region where the virus circulates, further TBEV strains from Estonia were isolated and characterized. In our analysis, we included the E protein sequences of 120 TBEV strains deposited in GenBank.

Materials and methods

Tick-borne encephalitis virus isolation

TBEV strains were isolated in 2002 and 2003 from adult *I. persulcatus* ticks collected in the south-eastern part of Estonia.

Tick pools were mortar ground and suspended in PBS. The numbers of ticks included in the pools are given in Table 1. For preparation of tick pool suspensions, 0.1 ml of PBS for each adult tick was used. Subsequently, each suspension was clarified by centrifugation at 3000 rpm for 10 min and the supernatants stored in aliquots at −70 °C. The samples were used for further virus isolation attempts in suckling mice. Samples were mixed in an equal volume of Medium-199 supplemented with neomycin (Gibco™, Scotland, UK). Two-day-old mice were inoculated intracerebrally with approximately 0.02 ml of the samples and observed for 14 days. Dead or moribund mice were removed, and 10% brain suspensions were prepared and stored at −70 °C until RNA extraction.

RNA extraction

RNA was extracted from brain suspensions of sick mice by the guanidinium thiocyanate-phenol-chloroform method using the TriPure RNA isolation system

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Table 1. Summary of the isolated tick-borne encephalitis virus strains from Estonia 2002–2003

Strain	Year	Place	*I. persulcatus* pool
Est 221	2002	North-eastern Estonia, Ida-Virumaa	14 M
Est 222	2002	North-eastern Estonia, Ida-Virumaa	13 F
Est 228	2002	South-eastern Estonia, Valgamaa	9 M
Est 274	2003	South-eastern Estonia, Tartumaa	15 F
Est 276	2003	South-eastern Estonia, Tartumaa	13 F
Est 293	2003	South-eastern Estonia, Tartumaa	17 F
Est 294	2003	South-eastern Estonia, Tartumaa	18 F
Est 312	2003	South-eastern Estonia, Tartumaa	11 M

F: adult females; M: adult males.

(Roche Diagnostics, Lewes, UK) according to the manufacturer's recommendations.

PCR amplification and sequencing

Sequences of the viral genome encoding partial E protein (nt 41–1250) of the Estonian TBEV strains were determined by direct sequencing of the PCR products. For the E gene, RT-PCR was performed using primers flanking nt 41–1312 (numeration is given according to the sequence of the strain Neudoerfl) as described earlier (Mickiene et al., 2001). PCR products were purified with GFX™ PCR DNA and Gel Band purification kit (GE Healthcare UK Limited, UK). For DNA sequencing reaction, BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Forest City, CA) was used according to the manufacturer's recommendations and sequenced on a 3100 DNA automated sequencer (Applied Biosystems). The obtained sequences were edited using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned with the corresponding region of sequences retrieved from GenBank (Table 2).

Phylogenetic analysis

Ninety TBEV strains available in GenBank, together with the strains isolated in this study, were included in a phylogenetic analysis of the partial E glycoprotein gene. The sequences of the strains Est 222 and 294 were identical to strains 221 and 293 and therefore were not included in the phylogenetic analysis. A region from nt 142–1247 of the E gene was selected as a majority of the published TBEV sequences covered this genomic region.

The maximum likelihood model was used for phylogenetic tree reconstruction, using the Tree Puzzle program (Schmidt et al., 2002), and 50,000 puzzling steps were applied using the Hasegawa-Kishino-Yano (HKY) model of substitution (Hasegawa et al., 1985). The transition/transversion ratio and nucleotide frequencies were estimated from the data set.

Predicted sequences of the E glycoprotein were produced in the BioEdit program. For the investigation of signature amino acids, in addition to 90 TBEV strains analyzed in the phylogenetic study, 30 TBEV strain sequences of shorter lengths were used (Table 2).

E glycoprotein structure analysis

The X-ray crystal structure of the TBEV glycoprotein E (Protein Data Base [PDB] entry 1SVB) (Rey et al., 1995) was used as a back-bone for the in silico reconstructions of a dimer molecule and models of S-TBEV strains isolated in Siberia and the Baltic countries. The homology modeling was initially performed using the automated homology modeling server SWISS-MODEL (http://swissmodel.expasy.org/) (Schwede et al., 2003) and verified with the molecular modeling and visualization programs. The protein structure analysis and graphic representation were made using the DeepView (http://swissmodel.expasy.org/spdbv/) (Guex and Peitsch, 1997) and VEGA programs (http://users.unimi.it/~ddl/) (Pedretti et al., 2002). The root mean square deviation of all non-hydrogen atoms of both viral strain models from the reference structure is less than 0.4 Å.

Results

A total of 8 strains belonging to the S-TBEV subtype were isolated from *I. persulcatus* ticks in Estonia in 2002 and 2003. The geographical origin of each strain is given in Table 2.

Phylogenetic analysis

On the phylogenetic tree (Fig. 1) based on the partial E glycoprotein gene (142–1247 nt), the Estonian strains were clustered with the previously identified TBEV strains of the S-TBEV subtype. Baltic strains, isolated in Latvia, Estonia, and Finland, formed a distinct Baltic lineage. The second lineage within the S-TBEV subtype consisted of strains isolated in Siberia (Novosibirsk,

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Table 2. Tick-borne encephalitis virus (TBEV) strains compared by phylogenetic analysis and amino acid alignment of the E glycoprotein

Strain	Geographical origin	Year of isolation	Source of isolate	Accession no.	References
Louping ill virus (LI), strain LI/917	England, UK	1997		X86786	Gao et al. (1997)
Pan	Moscow, Russia	1957	Human blood	AF091015	Ecker et al. (1999)
Lithuania-262	Lithuania	2001	Human blood	AJ414703	Mickiene et al. (2001)
TBE 4387	Southern Slovakia	1982	Bank vole	X76607	Labuda et al. (1994)
N256	Minsk, Belarus	Unknown	*I. ricinus*	AF091014	Ecker et al. (1999)
Latvia-11686	Latvia	2001	Human blood	AJ319582	Lundkvist et al. (2001)
Latvia-8369	Latvia	2001	Human blood	AJ319584	Lundkvist et al. (2001)
Kem I	Tatabanya, Hungary	1952	*I. ricinus*	AF091011	Ecker et al. (1999)
Absettarov	St. Petersburg region, Russia	1951	Human blood	AF091005	Ecker et al. (1999)
Kumlinge A52	Kumlinge, Finland	1959	*I. ricinus*	AY268437	Jaaskelainen et al. (2003)
Als. I	Alsace, France	1975	*I. ricinus*	AF091007	Ecker et al. (1999)
Hypr	Brno, Czech Republic	1953	Human blood	U39292	Wallner et al. (1996)
ZZ9	Zell/Ziller, Austria	1985	*I. ricinus*	AF091020	Ecker et al. (1999)
Scharl	Lower Austria, Austria	1956	Human brain	AF091017	Ecker et al. (1999)
Neudoerfl	Neudoerfl, Austria	1971	*I. ricinus*	genbank:U27495 genbank:U27495	Mandl et al. (1988)
Stara Ves	Stara Ves, Croatia	Unknown	Unknown	AF091018	Ecker et al. (1999)
Toro-2003	Sweden	2003	*I. ricinus*	DQ401139	Melik et al. (2007)
Latvia-12718	Latvia	2001	Human blood	AJ319586	Lundkvist et al. (2001)
Est3509	Estonia	2001	*I. ricinus*	DQ393778	Golovljova et al. (2004)
Est3053	Estonia	1996	*I. ricinus*	DQ393777	Golovljova et al. (2004)
Est3051	Estonia	1996	*I. ricinus*	DQ393775	Golovljova et al. (2004)
Ljub. I	Ljubljana, Slovenia	1993	Human blood	AF091012	Ecker et al. (1999)
235	Czech Republic	2006	Unknown	EF113081	GenBank
166	Czech Republic	2006	*I. hexagonus*	EF113079	GenBank
Latvia-9793	Latvia	2001	Human blood	AJ319585	Lundkvist et al. (2001)
Est3476	Estonia	2000	Human blood	DQ393776	Golovljova et al. (2004)
263	Czech Republic	1987	*I. ricinus*	U27491	Wallner et al. (1995)
274	Czech Republic	2006	Unknown	EF113083	GenBank
433	Czech Republic	2006	Unknown	EF116596	GenBank
282	Czech Republic	2006	Unknown	EF113087	GenBank
280	Czech Republic	2006	Unknown	EF113085	GenBank
Latvia-8110	Latvia	2001	Human blood	AJ319583	Lundkvist et al. (2001)
Iso 40	Schaffhausen, Switzerland	1975	*I. ricinus*	AF091009	Ecker et al. (1999)
K23	Karlsruhe, Germany	1975	*I. ricinus*	AF091010	Ecker et al. (1999)
Latvia 1-96	Latvia	2001	Human blood	AJ415565	Lundkvist et al. (2001)
Kokkola-9	Finland	2006	*I. persulcatus*	DQ451287	Jaaskelainen (2005)
Kokkola-8	Finland	2006	*I. persulcatus*	DQ451286	Jaaskelainen (2005)
Kokkola-26	Finland	2006	*I. persulcatus*	DQ451289	Jaaskelainen (2005)
Kokkola-25	Finland	2006	*I. persulcatus*	DQ451288	Jaaskelainen (2005)
Kokkola-102	Finland	2006	*I. persulcatus*	DQ451295	Jaaskelainen (2005)
Kokkola-86	Finland	2006	*I. persulcatus*	DQ451294	Jaaskelainen (2005)
Kokkola-118	Finland	2006	*I. persulcatus*	DQ451296	Jaaskelainen (2005)
Kokkola-39	Finland	2006	*I. persulcatus*	DQ451290	Jaaskelainen (2005)
Kokkola-79	Finland	2006	*I. persulcatus*	DQ451291	Jaaskelainen (2005)
Kokkola-84	Finland	2006	*I. persulcatus*	DQ451293	Jaaskelainen (2005)
Kokkola-81	Finland	2006	*I. persulcatus*	DQ451292	Jaaskelainen (2005)
Est54	Estonia	2000	*I. persulcatus*	DQ393773	Golovljova et al. (2004)
Est221	Estonia	2002	*I. persulcatus*		This paper
Est228	Estonia	2002	*I. persulcatus*		This paper
Est276	Estonia	2003	*I. persulcatus*		This paper

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Table 2. (*continued*)

Strain	Geographical origin	Year of isolation	Source of isolate	Accession no.	References
Est274	Estonia	2003	*I. persulcatus*		This paper
Est3535	Estonia	2001	*I. persulcatus*	DQ393774	Golovljova et al. (2004)
EK328	Estonia	1972	*I. persulcatus*	DQ486861	Romanova et al. (2007)
Est312	Estonia	2003	*I. persulcatus*		This paper
Est293	Estonia	2003	*I. persulcatus*		This paper
Zausaev	Russia	1985	Human brain	AF527415	Gritsun et al. (1993)
IR99-1m4	Irkutsk, Russia	1999	*I. persulcatus*	AB049349	Hayasaka et al. (2001)
IR99-2f7	Irkutsk, Russia	1999	*I. persulcatus*	AB049352	Hayasaka et al. (2001)
IR99-2m7	Irkutsk, Russia	1999	*I. persulcatus*	AB049351	Hayasaka et al. (2001)
IR99-1m1	Irkutsk, Russia	1999	*I .persulcatus*	AB049348	Hayasaka et al. (2001)
IR99-2m3	Irkutsk, Russia	1999	*I. persulcatus*	AB049350	Hayasaka et al. (2001)
IR99-2f13	Irkutsk, Russia	1999	*I .persulcatus*	AB049353	Hayasaka et al. (2001)
Aina	Irkutsk, Russia	1963	Human blood	AF091006	Ecker et al. (1999)
Vasilchenko	Novosibirsk, Russia	1969	Human blood	M97369	Gritsun et al. (1993)
D1283	Khabarovsk, Far-Eastern Russia	1998	Human brain	AB049347	Hayasaka et al. (2001)
Senzhang	China	1953	Unknown	AY174188	GenBank
Glybinnoe/2004	Primorsky krai, Russia	2004	Human brain	DQ862460	GenBank
Kam586/97	Hokkaido, Japan		*C. rufocanus*	AB237185	GenBank
T-blood	Perm, Russia	1939	Human blood	AF091019	Ecker et al. (1999)
RK1424	Latvia	1977	*I. persulcatus*	AF091016	Ecker et al. (1999)
N132	Vladivostok, Russia	1979	*I. persulcatus*	AF091013	Ecker et al. (1999)
Kita987/99	Hokkaido, Japan		*C. rufocanus*	AB237192	GenBank
KH98-2	Khabarovsk, Far-Eastern Russia	1998	*I. persulcatus*	AB022295	Hayasaka et al. (1999)
KH99-m9	Khabarovsk, Far-Eastern Russia	1999	*I. persulcatus*	AB049346	Hayasaka et al. (2001)
KH98-10	Khabarovsk, Far-Eastern Russia	1998	*I. persulcatus*	AB022297	Hayasaka et al. (1999)
KH98-5	Khabarovsk, Far-Eastern Russia	1998	*I. persulcatus*	AB022296	Hayasaka et al. (1999)
VL99-m11	Vladivostok, Russia	1999	*I. persulcatus*	AB049345	Hayasaka et al. (2001)
Est2546	Estonia	1996	*A. agrarius*	DQ393779	Golovljova et al. (2004)
Sofjin	Primorsky krai, Russia	1937	Human brain	X07755	Yamshchikov and Pletnev (1988)
DXAL5	North-eastern China		Unknown	AY178833	GenBank
205	Primorsky krai, Russia	1973	*I. persulcatus*	DQ989336	Safronov et al. (1990)
Crimea	Crimea, Ukraine	1987	*I. ricinus*	AF091008	Ecker et al. (1999)
Oh698/97	Hokkaido, Japan		*C. rufocanus*	AB237190	GenBank
Oh701/97	Hokkaido, Japan		*C. rufocanus*	AB237191	GenBank
Oh696/97	Hokkaido, Japan		*C. rufocanus*	AB237189	GenBank
Miz416/97	Hokkaido, Japan		*C. rufocanus*	AB237184	GenBank
Miz660/97	Hokkaido, Japan		*C. rufocanus*	AB237188	GenBank
Oshima	Hokkaido, Japan	1993	Cerebrospinal fluid	AB001026	Takashima et al. (1997)
O-I-1	Japan	1996	*I. ovatus*	AB022292	Hayasaka et al. (1999)
Kik629/97	Hokkaido, Japan		*C. rufocanus*	AB237187	Hayasaka et al. (1999)
973	Estonia	1986	*I. ricinus*	AF241774	GenBank
Vologda-2	Vologda region, Russia	1990	*I. persulcatus*	AF229364	GenBank
499	Western Siberia, Russia	1982	*I. persulcatus*	DQ394877	GenBank
396	Western Siberia, Russia	1981	*I. persulcatus*	DQ394880	GenBank

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Table 2. (*continued*)

Strain	Geographical origin	Year of isolation	Source of isolate	Accession no.	References
691	Western Siberia, Russia	1982	*I. persulcatus*	DQ394878	GenBank
228	Western Siberia, Russia	1981	*I. persulcatus*	DQ385498	GenBank
Stolby-4	Krasnoyarsk region, Russia	1988	*I. persulcatus*	AF231807	GenBank
Ural2005-1	Ural region, Russia	2005	*I. persulcatus*	EF029051	GenBank
Ural2005-2	Ural region, Russia	2005	*I. persulcatus*	EF029052	GenBank
Stolby-1	Krasnoyarsk region, Russia	1988	*I. persulcatus*	AF224666	GenBank
Botsad-1	Novosibirsk region, Russia	1986	*I. persulcatus*	AF224662	GenBank
367-81	Butyatia, Russia	1981	*A. speciosus*	AF241773	GenBank
Katun-3	Altai region, Russia	1985	*I. persulcatus*	AF236055	GenBank
Baikal-4	Irkutsk region, Russia	1986	*I. persulcatus*	AF229362	GenBank
Talakan-4	Amur region, Russia	1986	*I. persulcatus*	AF241772	GenBank
MDJ-01	China			AY217093	GenBank
Oshima 3-6	Hokkaido, Japan		Dog blood	AB022291	Hayasaka et al. (1999)
Oshima A-1	Oshima, Japan	1995	*A. speciosus*	AB022293	Hayasaka et al. (1999)
Oshima C-1	Oshima, Japan	1995	*C. rufocanus*	AB022294	Hayasaka et al. (1999)
Ural2005-3	Ural region, Russia	2005	*I. persulcatus*	EF029053	GenBank
P-202	Primorsky krai, Russia	1997	Human blood	DQ667009	Leonova et al. (2007)
P-69	Primorsky krai, Russia	2000	Human blood	DQ667007	Leonova et al. (2007)
P-73	Primorsky krai, Russia	1973	Human brain	DQ667008	Leonova et al. (2007)
Koltsovo19	Novosibirsk region, Russia		Human brain	AF540030	Ternovoi et al. (2003)
Koltsovo29	Novosibirsk region, Russia		Human brain	AF540032	Ternovoi et al. (2003)
Koltsovo-1	Novosibirsk region, Russia		Human brain	AF540029	Ternovoi et al. (2003)
Koltsovo23	Novosibirsk region, Russia		Human brain	AF540031	Ternovoi et al. (2003)
Koltsovo30	Novosibirsk region, Russia		Human brain	AF540033	Ternovoi et al. (2003)
Koltsovo31	Novosibirsk region, Russia		Human brain	AF540034	Ternovoi et al. (2003)
Hehcir	Habarovsk region, Russia	1985	*I. persulcatus*	AF229363	GenBank

Tomsk, and Irkutsk). While a geographical clustering of S-TBEV strains was observed, no such clustering could be seen with W-TBEV or FE-TBEV. Although well-supported genetic lineages are presented, no geographical clustering of the strains can be seen. For example, the Estonian strain Est2546 clustered together with the strain Sofjin, originating from Far East Russia, but not with the strain RK1424, isolated in neighboring Latvia (Fig. 1). The latter shares a common, more recent ancestor with strain N132 isolated in the Far East, and both of them share a more ancient ancestor with the T-blood strain originating from the Urals.

The nucleotide sequence maximum divergences were 5.7%, 7.5%, and 7.7% within the W-TBEV, FE-TBEV, and S-TBEV subtypes, respectively. The degree of nucleotide sequence differences between the subtypes ranged from 12.9% to 17%. Although the strains showed a high degree of similarity in the nucleotide sequences of the E glycoprotein, strains of FE- and S-TBEV subtypes were more heterogeneous when compared to the W-TBEV subtype. This could be due to a geographically more dispersed distribution of the FE- and S-TBEV strains than that of the W-TBEV strains.

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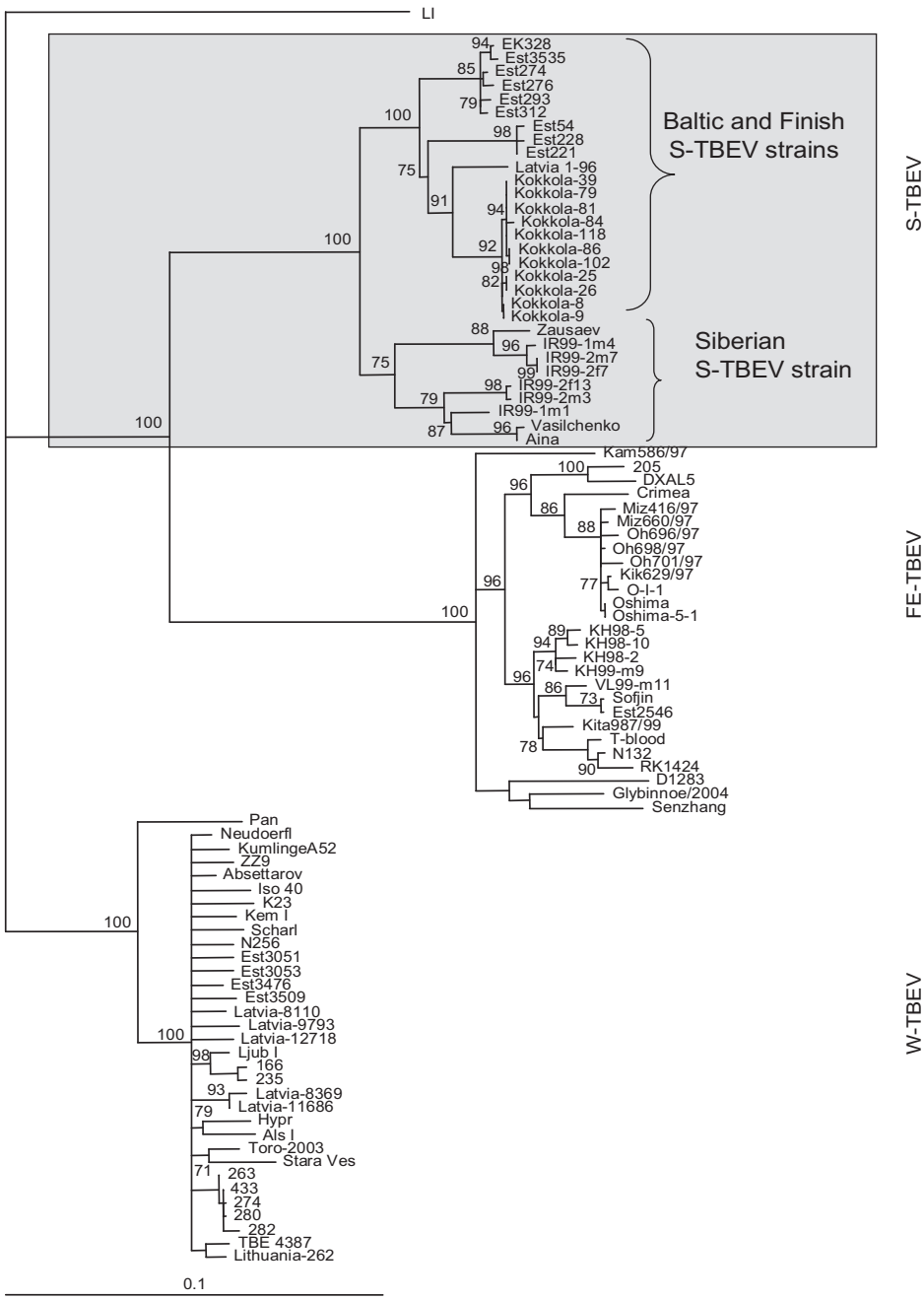

Fig. 1. Phylogenetic tree (maximum likelihood) based on E glycoprotein encoding sequences (nt 142–1247). Bootstrap values > 70% (derived from 50,000 replicates neighbor-joining trees estimated under ML substitution model) are shown. For information regarding the tick-borne encephalitis virus strains see Table 2. (S: Siberian subtype; FE: Far-Eastern subtype; W: Western subtype.)

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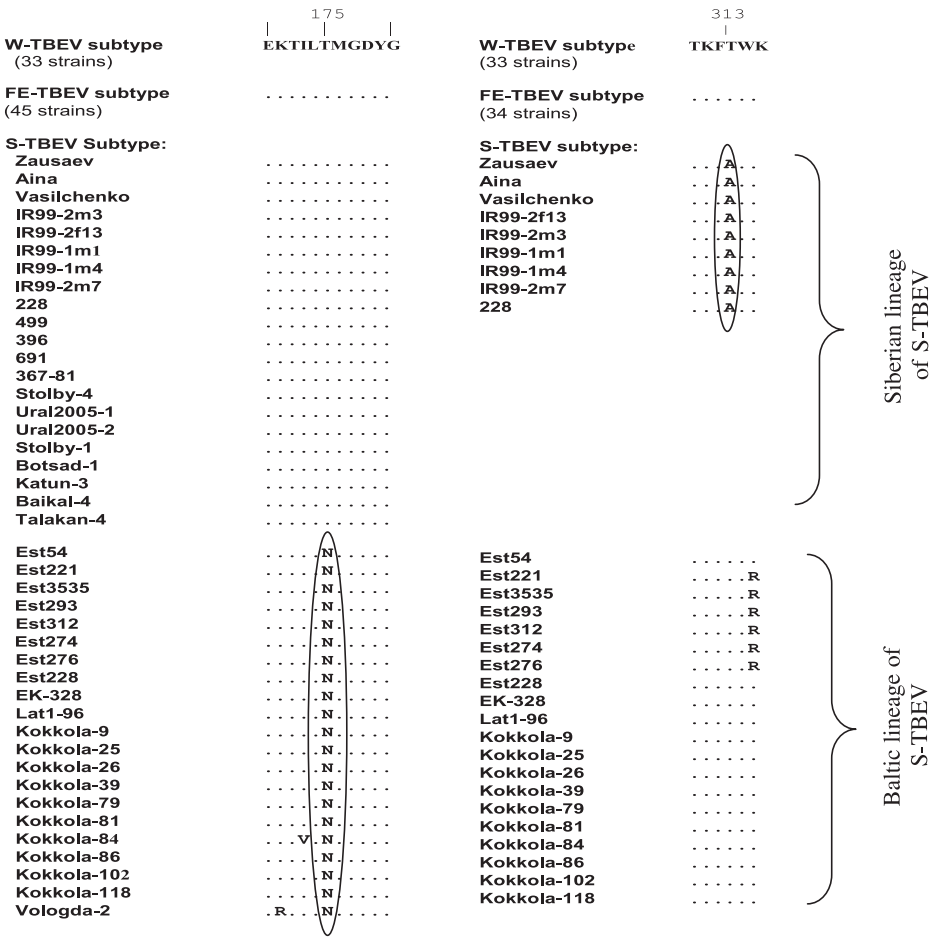

Fig. 2. Comparison of signature amino acids of the E glycoprotein at positions 175 and 313 within the S-TBEV subtype. Amino acid alignment of the strain Neudoerfl is shown on top and dots indicate identical amino acids. For information regarding the tick-borne encephalitis virus strains see Table 2. (S: Siberian subtype; FE: Far-Eastern subtype; W: Western subtype.)

Amino acid analysis

Analysis of the deduced amino acid alignment of the partial E glycoprotein revealed in general the same signature amino acids for all subtypes as previously described (Ecker et al., 1999) and new signature amino acids within S-TBEV. We found that the earlier published signature amino acids for the S-TBEV subtype (Ecker et al., 1999) at positions 119, 279, and 350 were not present in the majority of the S-TBEV strains. At position 313, all Siberian strains of the S-TBEV subtype had a unique Thr/Ala substitution and differed from Baltic strains of the same subtype and from the strains belonging to the FE- and W-TBEV subtypes (Fig. 2). All Baltic strains of the S-TBEV subtype and the strain Vologda-2 isolated in the European part of Russia (not included in the phylogenetic analysis due to a short published nucleotide sequence) had a unique Thr/Asn substitution at position 175, previously not referred to as a signature amino acid (Fig. 2). Comparison of the deduced E glycoprotein amino acid sequences within the S-TBEV confirmed that these strains form two groups. The first group included strains isolated in Siberia and the Far East and had signature amino acid Ala at position 313, while the second group included Baltic strains and a strain from the European part of Russia with signature amino acid Asn at position 175.

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Protein structure modeling

The protein modeling and structure analysis of the molecular surface of the protein E dimer molecule showed that both signature amino acids 175 and 313 are located at the surface, at one side of the dimer and close to each other (Fig. 3a). The span over the molecule surface between them was about 35 Å, and the proximity of these residues did not exclude their participation in one binding site. For both substitutions, Thr175Asn as well as Thr313Ala, there were no sterical obstacles since in both cases the side chains are oriented to the exterior of the protein molecule and are comparable in volume.

Signature amino acid Ala313 in the Siberian S-TBEV strains was oriented outwards from the TBE dimer surface as well as Asn175. Residue 313 belongs to the β sheet H, and the substitution had no influence on the presence of hydrogen bonds within the main chain. Geometrical analysis of the molecular surface around amino acid 313 suggested the presence of a cleft between this residue and a loop formed by residues 308–311, proposed as a putative flavivirus receptor binding site (Mandl et al., 2000) (Fig. 3b).

E protein modeling suggested that at position 175, the differences in polar propensities of Thr and Asn could provide a structural difference between the E proteins of Siberian and Estonian lineages of S-TBEV. E protein modeling showed that Thr175 was located in the β strand of all antiparallel β sheet B, and the main chain atoms formed the hydrogen bond with Asp181 in the next antiparallel β strand of the same sheet B. Thr175 and Asp181 were connected by the short loop containing an α-helical turn 177–179 (Fig. 3c). This β hairpin stabilizes the spatial orientation of residues 177–179 on the molecule surface towards the 308–311 residue loop. Thus, strains of Siberian S-TBEV had an additional hydrogen bond between Thr175 and Asp181, which is lacking in the Baltic strains.

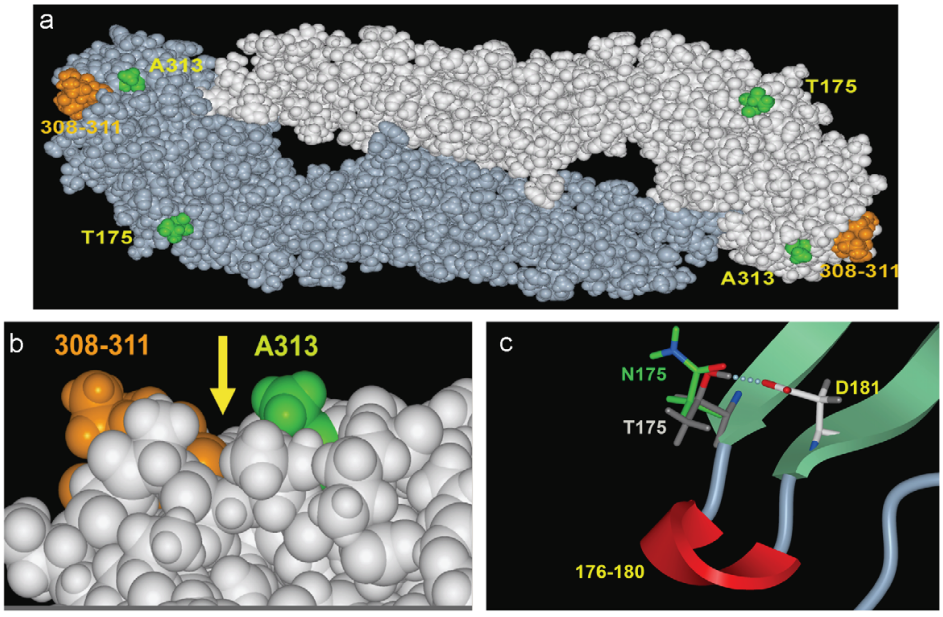

Fig. 3. The E glycoprotein of the tick-borne encephalitis virus (TBEV). (a) Surface model. The two subunits of the dimer are colored white and grey, respectively. The amino acids 175 and 313 that distinguish the Baltic from Siberian lineages of the Siberian TBEV are coloured green and labeled A313 and T175. The putative receptor-binding site, the 308–311 loop, is coloured orange. (b) Model representation of signature amino acid Ala313 for Siberian lineage S-TBEV on the surface of the E glycoprotein. Atoms are shown in balls, Ala313 is colored green and labelled A313, the 308–311 loop is coloured orange and labelled 308–311. The arrow indicates a cleft on the surface between residues 308–311 and 313. (c) Model representation of signature amino acid 175 for the Baltic lineages of S-TBEV strains on the surface of the E glycoprotein. The backbone is shown by ribbon representation; two β strands of antiparallel β sheet B in green, connected by labelled loop 176–180 containing the helical turn 177–179 and shown by red ribbon. The side chains of signature amino acid residues 175 are given in stick representation and coloured: green – Asn for the Baltic lineage of S-TBEV (N175) and grey – Thr for the Siberian lineage (T175). Partly charged heavy atoms of residue polar groups are indicated with standard colors: oxygen with red and nitrogen with blue. Side chain of residue 181 is shown in stick representation and labeled as D181. The hydrogen bond between Asp181 and Thr175 in all TBEV subtypes, which is missed in the Baltic lineage of S-TBEV, is depicted.

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Discussion

In spite of a high degree of genetic similarities between TBEV strains within, as well as between, the subtypes, we found a clear geographical separation of strains belonging to the S-TBEV subtype. Thus, it is possible to suggest that no mixing occurs between these two lineages at present. Within this subtype two lineages are reported: (i) a Baltic lineage, comprising strains from the Baltic countries, Finland, and the European part of Russia, and (ii) a Siberian lineage, comprising strains from the Far East and Siberia. This geographical clustering of S-TBEV was also supported by a phylogenetic analysis on the amino acid level by the presence of unique signature amino acids at positions 175 and 313 for Baltic and Siberian strains, respectively. It has previously been demonstrated that each subtype of TBEV has specific signature amino acids (Ecker et al., 1999), but no specific substitutions according to geographical location, time, or source of isolation within subtypes were reported. The vector of S-TBEV as well as of the FE-TBEV subtype is *I. persulcatus*, and strains belonging to these subtypes are isolated from the Far East to the Baltic countries in Europe, i.e. the whole territory of the vector distribution. However, within the FE-TBEV subtype, strains isolated in Europe, RK1424, and Est2546, are closely related to strains from the Far East and have not formed their own European or Baltic cluster. These data could suggest a different evolutionary history of the S- and FE-TBEV subtypes, in spite of the same arthropod vector.

It has been suggested that flaviviruses, including TBEV, have dispersed and evolved in a cline across the Eurasian continent in an east-to-west direction during the last few thousand years (Zanotto et al., 1995). The S- and FE-TBEV strains share a common ancestor, and are more closely related to each other than to W-TBEV (Fig. 1). The time of divergence of the S- and FE-TBEV subtypes was estimated at approximately 1700–2100 years ago (Hayasaka et al., 1999), and thus, the divergence within the S-TBEV subtype should have occurred later. The mode of such a fast dispersal of flaviviruses through the Eurasian continent is still unclear. The distribution and spread of TBEV is closely associated with their vectors and hosts. Although ticks may be transported significant distances on mammals and birds, they do not move horizontally in their natural environment.

For certain other viruses, e.g. hantaviruses, it was demonstrated that their evolutionary history was closely related to the evolutionary history of their rodent hosts (Plyusnin et al., 1996). Geographical clustering of hantaviruses in Europe reflects a route of postglacial colonization of Europe by rodents from their glacial refuges. The retreat of the last glaciation approximately 10,000 years ago allowed for the formation of the present flora and fauna and the association of ticks with their rodent hosts in the forests of the northern hemisphere. Little is known about the phylogenetic relationships of the *Ixodes* ticks and routes of postglacial colonization of Europe and the Far East. However, on the basis of morphological, seasonal adaptation, and paleogeographical data, *I. persulcatus* is a younger species compared to *I. ricinus* (Filippova, 1985). It was also suggested that ancestors of *I. persulcatus* and *I. ricinus* have been widely distributed in Neocene. However, with glaciation, their spread was halted and ancestor ticks survived in different glacial refuges, probably together with their hosts, rodents, and wild mammals (Filippova, 1985). It could be possible that the ancestors of Baltic and Siberian strains of the S-TBEV subtype were separated in different glacial refuges, and then colonized Europe and Siberia on different routes together with ticks. In this case, *I. persulcatus* from Europe and Siberia also should probably be distinguishable on the genetic level. This hypothesis does, however, not support the cline concept of TBEV distribution (Zanotto et al., 1995), which suggests that radiation of the cline started in the Far East towards the west relatively recently, around 2500 years ago, when the postglacial colonization of Europe, Siberia, and the Far East was already completed.

The boundaries between the two lineages of S-TBEV strains probably lie in the Ural Mountains. Strains belonging to the Baltic lineage of S-TBEV were isolated to the west and strains belonging to the Siberian lineage to the east of the Ural Mountains. While it was previously demonstrated that the Ural Mountains phylogeographically separated certain rodent species populations into different lineages (Fedorov et al., 1999; Brunhoff et al., 2003; Haynes et al., 2003), other mammal species did not show this kind of separation (Oshida et al., 2005). Extrapolation of data for mammals is however difficult on *I. persulcatus* and TBEV, since several kinds of vertebrate hosts are involved in the life cycle of the virus during the s everal stages of tick development. The phylogenetic relationships of viruses not only depend on vector and virus relationships, but also on vector–vertebrate host relationships.

The functional roles of the signature amino acid substitutions are still unknown. The fact that most of these substitutions are situated on the upper or lateral surfaces of the E glycoprotein suggests that these amino acids may be involved in cell surface binding (Ecker et al., 1999). It has also been suggested that the signature amino acids may reflect the species of ticks that act as vectors for TBEV (Gritsun et al., 1993). The signature amino acids may be responsible for TBEV pathogenicity, as it was reported that different subtypes of TBEV cause different clinical forms of TBE, i.e. FE-TBEV subtype strains cause a more severe form of disease in

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humans with a reported fatality rate of 5–20% (Dumpis et al., 1999), while W-TBEV subtype strains cause a relatively mild, typically biphasic illness with a low fatality rate (less than 1%) (Gresiková and Kaluzova, 1997; Kaiser, 2002; Mickiene et al., 2002). Although the S-TBEV strains isolated in Siberia caused chronic TBEV infection in 1–3% of all TBEV cases and also nonparalytic, febrile forms of encephalitis (Monath and Heinz, 1996; Gritsun et al., 2003a, b; Poponnikova, 2006), chronic TBE has not been reported in Europe to date.

Analysis of the E glycoprotein structure by homology modeling suggested structural features for the Baltic and Siberian lineages of S-TBEV strains at positions 313 and 175. We found that amino acid substitution at position 313 in the Siberian S-TBEV strains was located on the upper-lateral surface of domain III. It was suggested that this domain is responsible for binding tick-borne but not mosquito-borne flaviviruses to their target cells (Bhardwaj et al., 2001). Also attenuation of TBEV has been achieved by site-specific mutagenesis in this region of the E glycoprotein (Mandl et al., 2000). Different hydrophobicity and polarity of Thr/Ala residues may influence the binding properties of the cleft formed by residue 313 and the 308–311 residue loop, which is a putative cell receptor-binding site. The other unique signature amino acid at position 175 within the Baltic lineage of S-TBEV strains demonstrated the lack of a hydrogen bond between Thr175 and Asp181 side chains (Fig. 3), making the 177–179 loop more flexible. The biological role of these findings is unclear, but the fact that both signature amino acids are situated on the viral surface close to the receptor-binding site, may constitute indirect evidence of the involvement of these signature amino acids in viral functional processes.

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PUBLICATION II

Identification of *Anaplasma phagocytophilum* in tick populations in Estonia, the European Part of Russia and Belarus

Katargina, O., Geller, J., Alekseev, A., Dubinina, H., Efremova, G., Mishaeva, N., Vasilenko, V., Kuznetsova, T., Järvekülg, L., Vene, S., Lundkvist, Å. and Golovljova, I.

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Identification of *Anaplasma phagocytophilum* in tick populations in Estonia, the European part of Russia and Belarus

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Abstract

Anaplasma phagocytophilum is associated with diseases of goats, sheep, cattle, dogs and horses. In the beginning of the 1990s it was identified as a human pathogen, causing human granulocytic anaplasmosis (HGA) in the USA, Europe and the far east of Russia. *A. phagocytophilum* is maintained in nature in an enzootic cycle including ticks as the main vector and a wide range of mammalian species as reservoirs. *Ixodes ricinus* and *I. persulcatus* ticks were collected in Estonia, Belarus and the European part of Russia and screened for the presence of *A. phagocytophilum* by real-time PCR. Positive samples were found only among *I. ricinus*, in 13.4% in the European part of Russia, 4.2% in Belarus, 1.7% in mainland Estonia and 2.6% on Saaremaa Island. Positive samples were sequenced for partial 16S rRNA, *groESL* and *ankA* genes and phylogenetic analyses were performed. The results showed that *A. phagocytophilum* circulating in Eastern Europe belongs to different *groESL* lineages and 16S rRNA gene variants and also consists of variable numbers of repetitive elements within the *ankA* gene.

Keywords: 16S rRNA gene, *Anaplasma phagocytophilum*, *ankA* gene, *groESL* operon, *Ixodes persulcatus*, *Ixodes ricinus*, prevalence, ticks
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Introduction

Anaplasma phagocytophilum is an obligate intracellular bacterium that infects leucocytes and multiplies in cytoplasmic vacuoles [1]. It has been identified as a human pathogen, causing human granulocytic anaplasmosis (HGA), since 1994, when the first human case was reported in the United States [2]. In Europe, the numbers of human cases are continuously increasing and HGA has been reported from Austria, Italy, the Netherlands, Spain, Poland, Slovenia, Sweden, Estonia and the far east of Russia [2]; however, to our knowledge no HGA cases in the European part of Russia and Belarus have been reported up to date. In spite of absent or low numbers of clinical cases in Europe, HGA-seroconversion was reported in 11.3% of the population in Belarus (Mishaeva, unpublished data), in approximately 6% of the population in European countries [3] following tick bites, and in 8.3% of Slovenian forestry workers in areas endemic for tick-borne diseases [4].

A. phagocytophilum is maintained in nature in an enzootic cycle including ticks as the main vector and a wide range of mammalian species as reservoirs. *I. ricinus* ticks act as the main vector of *A. phagocytophilum* in Europe, while *I. persulcatus* is the vector in Asia, the Urals, Siberia, the Far East, and in the Russian Baltic region. In Europe, large wild mammals have been suggested to act as reservoir hosts, amongst them mainly roe deer and red deer [5], but also small mammals and rodents [6]. Different strains, variants and lineages of *A. phagocytophilum* circulate widely in natural foci and display different host and/or vector tropism and pathogenicity [5,7–9].

For genetic characterization of *A. phagocytophilum*, small subunits of rRNA (16S rRNA), a heat-shock protein (*groESL*) or the *AnkA* protein (*ankA*) genes have been utilized. Although the similarities between the 16S rRNA gene sequences are high, several 16S gene rRNA variants have been reported [9–11]. Sequences of the *groESL* and the *ankA* genes are more diverse and thereby more reliable for genetic classification [5,12].

The objectives of the present study were detection of *A. phagocytophilum* in ticks collected in Estonia, the European part of Russia and Belarus and genetic characterization by sequencing of the partial 16S rRNA gene, the *groESL* operon and the *ankA* gene.

Materials and Methods

Tick collection

In 2006–2008, *I. ricinus* and *I. persulcatus* ticks were collected by flagging vegetation at 14 sites on mainland Estonia, ten sites on Saaremaa island, four sites in the European part of Russia (St Petersburg surroundings, Curonian Spit, Borok and Tcherepovets) and at seven sites in Belarus (Minsk surroundings) (Fig. 1). Tick species were identified by morphological criteria independently by two entomologists. Ticks were homogenized in 300 μl of PBS by TissueLyzer (Retsch, Haan, Germany). Two hundred microlitres of suspensions were used for DNA extraction.

DNA extraction

DNA was 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 included as a negative control for every DNA preparation set.

Real-time PCR

Samples were screened for the presence of *A. phagocytophilum* by real-time PCR using ApMSP2f and ApMSP2r primers, and an ApMS2-FAM probe as described by Courtney [13].

PCR amplification of the partial *groESL*, the 16S rRNA and the *ankA* genes

Twenty-one samples positive for *A. phagocytophilum* by real-time PCR were chosen for the partial *groESL*, the 16SrRNA and the *ankA* gene amplification.

Amplification of the partial *groESL* operon PCR was performed with primers HS1 and HS6 for the first PCR and HS43 and HSVR for the nested PCR as described by Sumner [14] and Lotric-Furlan [15].

The partial 16S rRNA gene was amplified with primers Ehr1 and Ehr6 for the first PCR and Ehr7 and Ehr8 for the nested PCR as described by Rar [16,17].

The 3′ portion of the *ankA* gene was amplified as described by von Loewenich and Massung [12,18]. Primers 1F and 4R1 were used for the first PCR and the product of this reaction was used for nested reactions with the following sets of primers: 1F1 and 4R1mod, 2F1 and 2R1, and 3F1 and 4R1mod.

To avoid contamination, the extraction of DNA, the preparation of the master mixes and the PCR were performed in separate rooms.

DNA sequencing

PCR products were purified by the GFX™ PCR DNA and Gel Band purification kit (GE Healthcare UK Limited, UK). The BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Forest City, CA, USA) was used for the DNA sequencing reaction, according to the manufacturer's recommendations, followed by sequencing on a 3100 DNA automated sequencer (Applied Biosystems). The obtained sequences were edited using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and aligned by the ClustalW multiple alignment option with the corresponding region of sequences retrieved from GenBank.

Phylogenetic analysis

The Maximum Likelihood model was used for phylogenetic tree reconstruction of the partial *groESL* operon (1160 bp) and partial *ankA* (1591–1672 bp) using the Tree Puzzle program and 10 000 puzzling steps were applied using the Hasegawa–Kishino–Yano (HKY) model of substitutions. The transition/transversion ratio and nucleotide frequencies were estimated from the dataset.

Identical sequences were excluded from the phylogenetic analyses and the sources and places of their origin are shown within parenthesis in the phylogenetic trees (Figs 2 and 3).

Results

A total of 3976 ticks were collected in Estonia, among them 2474 *I. ricinus* from mainland Estonia, 345 *I. ricinus* from Saaremaa island and 1157 *I. persulcatus* from south-eastern Estonia. In Russia, 477 ticks were collected, among them 82 *I. ricinus* from Curonian Spit and 395 *I. persulcatus* from the European part of Russia (St Petersburg, Borok, Tcherepovets). In Belarus, a total of 187 of *I. ricinus* were collected from the Minsk surroundings.

LOW RESOLUTION FIG

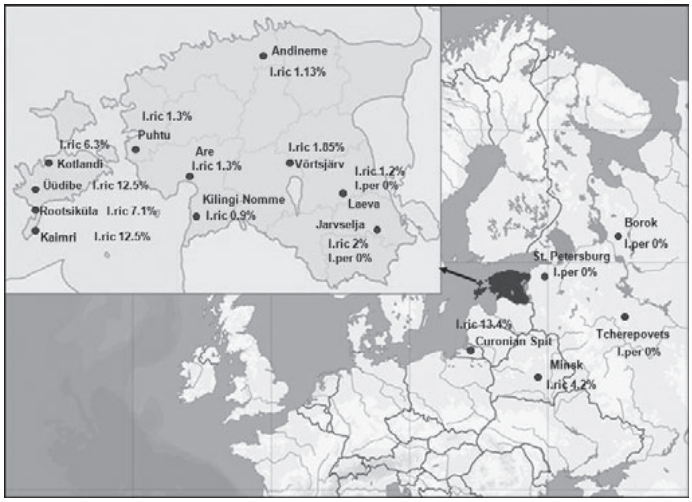

FIG. 1. Map of Estonia, the European part of Russia and Belarus where positive ticks were collected; percentages show the prevalence of *A. phagocytophilum* in the tick population. I. ric, *Ixodes ricinus* ticks; I. per, *Ixodes persulcatus* ticks. 16

In our study *A. phagocytophilum* was found only in *I. ricinus* ticks even at regions where *I. ricinus* and *I. persulcatus* co-circulate. The prevalence of *A. phagocytophilum* in ticks was detected by real-time PCR and we found that 1.7% of *I. ricinus* (42/2 474) were infected in seven out of 14 geographical sites in mainland Estonia. On Saaremaa island *A. phagocytophilum* was detected in nine out 345 ticks, with an overall prevalence of 2.6%. However, positive ticks were found only at four sites (Kamri, Kotlandi, Rootsiküla, Üübide), where the prevalence varied from 6.3% to 12.5%. In Russia, 11 out of 82 (13.4%) *I. ricinus* collected on the Curonian Spit were found to be positive. In Belarus, *A. phagocytophilum* was detected in eight out of 187 ticks (4.2%) (Fig 1).

A total of 1262 bp of the *groESL* heat shock operon were analysed for the 21 samples found positive by the real-time PCR. The nucleotide identity of the sequenced samples was high, from 98.8% to 100%. By analysing the first 402 predicted amino acids of the groEL protein sequence we found amino acid substitutions at position 139 (Glu → Arg in two Estonian samples) and at position 398 (Glu → Gly in one of the Belarusian samples). According to the substitutions at amino acid position 242, all analysed sequences (21 from this study and 38 retrieved from GenBank) were divided into two groups, one with serine (lineage 1) and another with alanine (lineage 2) at this position. The splitting of *A. phagocytophilum* into two genetic lineages was confirmed by phylogenetic analysis (Fig 2). On the phylogenetic tree based on the partial *groESL* operon sequences (1160 bp), lineage 1 subdivided with high bootstrap support into the European and New World lineages, while lineage 2 included only *A. phagocytophilum* from Europe. According to the information available in GenBank, *A. phagocytophilum* pathogenic for humans or domestic animals (horses, dogs, sheep) have to date been reported only within lineage 1. The sequences analysed in the present study belonged to both *A. phagocytophilum* genetic lineages; moreover, lineages 1 and 2 co-circulated at the same sites in Estonia, Belarus and in the Russian part of the Curonian Spit. 7

The partial 16S rRNA gene (1168 bp) was sequenced for 15 samples out of 21 found positive for the *groESL* operon gene. We found that the sequences of *A. phagocytophilum* from Estonia, Belarus and Russia shared a high degree of similarity (98.9–100%). Ten sequences of the analysed samples were identical, and only five samples could be distinguished from each other. A variable region near the 5′end of the 16S rRNA gene at position 76–84 was used for distinguishing the *A. phagocytophilum* variants [9,11,19,20]. Four variants of *A. phagocytophilum* were revealed in our study (Table 1). Variant 1 was found in eight samples collected on mainland Estonia, the Russian part of the Curonian Spit and in Belarus; variant 2 was found in one sample from the Estonian island Saaremaa; variant 3 was found in five samples from mainland Estonia, Saaremaa island and the Curonian Spit (Russia); and variant 4 was found in only one sample from Belarus.

In eight of 21 samples positive for the *groESL* gene, the partial *ankA* gene could be amplified. Sequencing of PCR products revealed different lengths of the 3′portion of the *ankA* gene: 1591 bp for the samples from Belarus (BelBmi11 and Bel6-22- 8

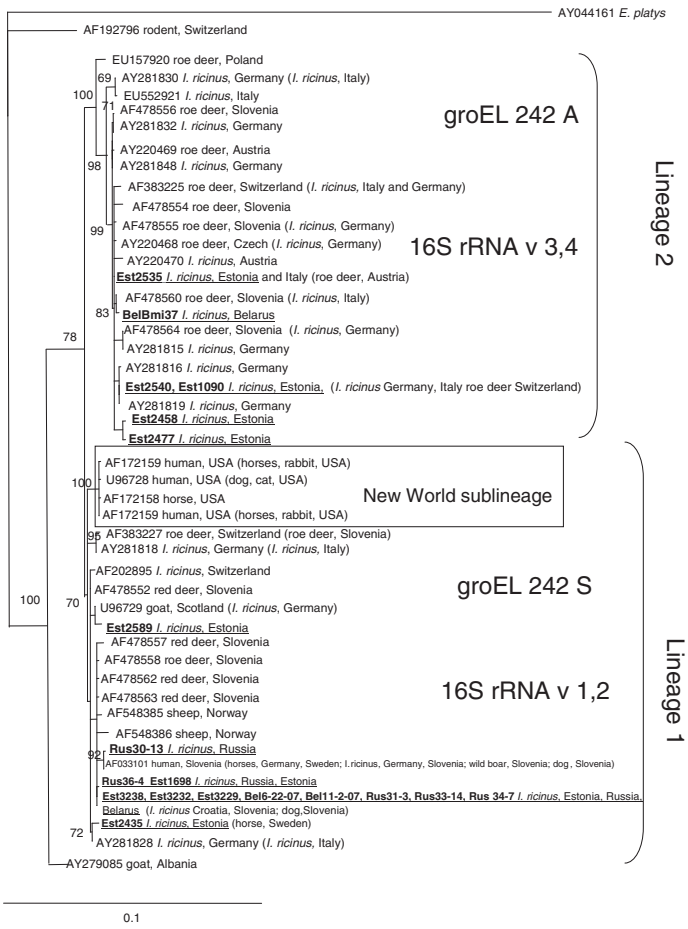

FIG. 2. Phylogenetic tree (Maximum Likelihood) based on the partial sequence of *groESL* operon (nt 423–1679). Only bootstrap values >70% (derived from 10 000 replicates of neighbour-joining trees estimated under the ML substitution model) are shown. Source and place of detection of identical sequences are shown in parentheses. Samples sequenced in the present study are indicated in bold and underlined.

07), 1642 bp for the samples from Estonia and Russia (Bel11-2-07, Rus34-7, Rus30-13, Est3238), 1672 bp for the Belarusian samples (BelBmi37), and 1717 bp for the Estonian sample (Est3239). Sequencing revealed four distinct models of *ankA* gene organization (Fig 4). In four samples (Bel11-2-07, Rus34-7, Rus30-13 and Est3238) 25-, 27- and 17-amino-acid repeats were found as single copies and 11-amino-acid repeats in two copies. A similar organization of the *ankA* gene has been described for samples from humans (Sweden and Slovenia) [18], horses (Germany and Switzerland), dogs (Germany and Denmark) and sheep (Germany) (F. D. von Loewenich, unpublished data, GenBank). Two samples (Bel6-22-07 and BelBmi11) showed one copy of 25- and 27-amino-acid repeat, no 17-amino-acid element and two copies of 11-amino-acid repeats. The same *ankA* gene sequence was found only in one sample from a dog in Austria (F. D. von Loewenich, unpublished data, GenBank accession number GU236826). The sequence for Estonian sample, Est3239, included two copies of a 25-amino-acid repeat, one copy of 27- and 17-amino-acid elements and two copies of an 11-amino-acid repeat. A similar organization has been detected in *A. phagocytophilum* from ticks in Germany [12], and in dogs from Germany and Switzerland (F. D. von Loewenich, unpublished data, GenBank). The *ankA* gene sequence of sample BelBmi37 consisted of one copy of 25-, 27-and 11-amino-acid elements, and a 33-amino-acid insertion located directly adjacent to and upstream from a 27-amino-acid repeat. A similar *ankA* gene organization has been described for *A. phagocytophilum* from *I. ricinus* collected in

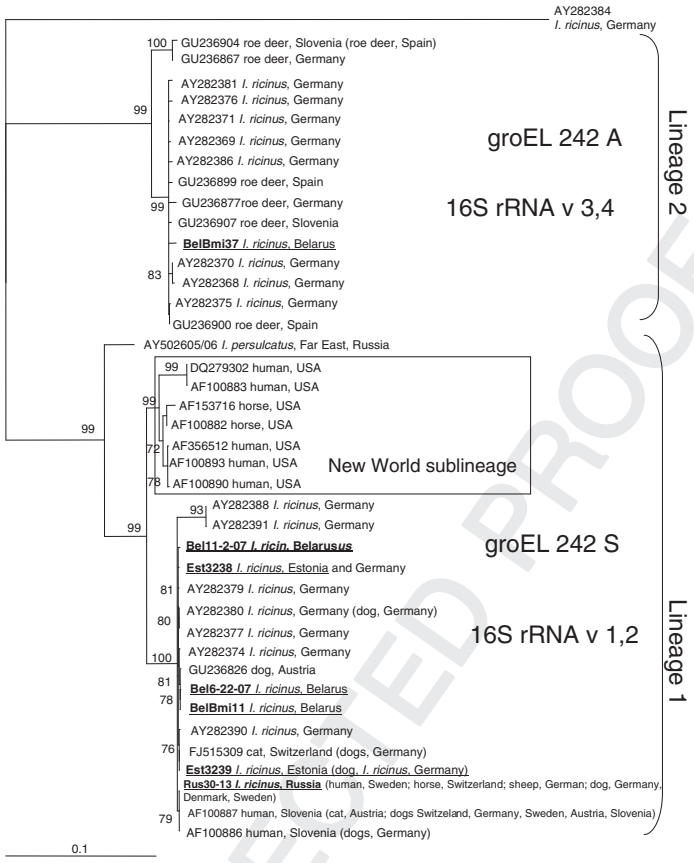

FIG. 3. Phylogenetic tree (Maximum Likelihood) based on the partial sequence of *ankA*. Only bootstrap values >70% (derived from 10 000 replicates of neighbour-joining trees estimated the under ML substitution model) are shown. Source and place of detection of identical sequences are shown in parentheses. Samples sequenced in the present study are indicated in bold and underlined.

TABLE 1. 16S rRNA gene variants detected in *I. ricinus* ticks collected in Estonia, the European part of Russia and Belarus 15

			Nucleotide positions								
No. of samples	**Place of tick collection**	**16S variant**	**76**	**77**	**78**	**79**	**80**	**81**	**82**	**83**	**84**
Est3229, Est3238	Mainland of Estonia	1	A	A	A	G	A	A	T	A	G
Rus30-10, Rus30-13, Rus34-7	Curonian Split, Russia										
Bel 11-2-07, Bel 6-22-07, Bel Bmi11	Minsk, Belarus										
Est2589	Estonia, Saaremaa	2	A	A	A	G	A	A	T	A	**A**
Est1090	Mainland of Estonia	3	**G**	A	A	G	A	A	T	A	**A**
Est2477, Est2535, Est2540	Estonia, Saaremaa										
Rus 29-12	Curonian Split, Russia										
Bel Bmi37	Minsk, Belarus	4	**G**	A	A	G	A	A	T	A	G

Germany [12] and samples from roe deers in Germany, Slovenia, Spain and Norway (F. D. von Loewenich, unpublished data, GenBank). *A. phagocytophilum* samples demonstrated 99.8–100% similarity on the nucleotide level within each *ankA* gene sequence type, while between different *ankA* sequence types the similarity was only 64.6–95.5%. We found that *A. phagocytophilum* with a different *ankA* gene organization may co-circulate in the same geographical region, for example in Estonia (Est3238 and Est3229) and in Belarus (Bel11-2-07, Bel6-2207 and BelBmi37) (Fig 4). 10

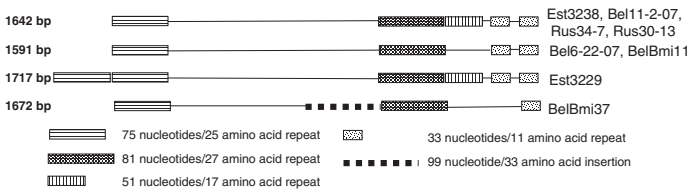

FIG. 4. Schematic presentation of the variable numbers of repetitive elements within the *ankA* gene detected in this study.

On a phylogenetic tree based on the partial *ankA* gene, the sequences of *A. phagocytophilum* formed two lineages (Fig 3) and the distinction between lineages 1 and 2 corresponded well with the lineage splitting based on the *groESL* phylogenetic tree. Samples of *A. phagocytophilum* sequenced in both regions (*ankA* and *groESL* genes) had serine or alanine for lineage 1 and 2, respectively, at amino acid position 242 of the groEL protein. Similar to the phylogenetic tree based on the partial *groESL* operon, lineage 1 divided into European and New World sublineages and lineage 2 consisted of *A. phagocytophilum* sequences detected in Europe.

A. phagocytophilum within lineage 1 and sequenced for the *groESL*, *ankA* and the 16S rRNA genes were classified as 16S rRNA gene variants 1 and 2, while variants 3 and 4 were detected within lineage 2.

Discussion

In the present study positive samples were found only among *I. ricinus*, despite the fact that *A. phagocytophilum* has been detected in 1% of *I. persulcatus* in a neighbouring region (St Petersburg, Russia) [21]. In Siberia and the Far East, where *I. persulcatus* is the main vector for *A. phagocytophilum*, the prevalence varied from 1% in the Ural to 9.6% in Japan [16,22]. However, *A. phagocytophilum* has previously been reported simultaneously in *I. persulcatus* and *I. ricinus* at geographical sites where the two tick species are distributed. Moreover, sequences of the *A. phagocytophilum* p44/msp2 gene indicated that there are no differences between sequences derived from *I. ricinus* and *I. persulcatus* [23]. In Estonia, even at two sites in south-eastern Estonia, where both species of ticks co-circulate and *I. ricinus* constitutes the minority (10–20%) of the tick population, we found *A. phagocytophilum* only in *I. ricinus*; thus, the role of *I. persulcatus* for the circulation of *A. phagocytophilum* in Estonia is unclear and further tick collections are necessary.

In present study *A. phagocytophilum* was detected in 0.9–13.4% of *I. ricinus* ticks. The reported prevalence in Europe also varied significantly from 0.3% in western France [24] to 17.1% on Norwegian islands [25]. As described earlier, ticks collected at different time-points at the same locality had different rates of *A. phagocytophilum* infection [26], and differences in prevalence may reflect seasonal or annual variation of tick infection rates, or different sampling, storage and methodical approaches. Therefore comparisons of the prevalence between various countries or different areas should be performed with caution.

Direct comparison of the 16S rRNA genetic variants of *A. phagocytophilum* lineages based on the *groESL* and *ankA* genes is difficult due to the limited number of simultaneously available *A. phagocytophilum* sequences for all three genes. However, eight samples of *A. phagocytophilum* sequenced in all three regions (16S rRNA, *groESL* and *ankA*) in the present study and 25 sequences published previously [12] showed that *A. phagocytophilum* sequences formed similar lineages on the *groESL* and the *ankA* gene phylogenetic trees. Division into lineages corresponded also with the 16S rRNA genetic variants; variants 1 and 2 clustered within lineage 1, variants 3 and 4 within lineage 2. The *A. phagocytophilum* belonging to lineage 1 were widely distributed in Europe and the USA and have been detected in humans, ticks, sheep, goats, horses, dogs, red deer and roe deer. Sequences belonging to lineage 2 have been detected only in roe deer and *I. ricinus* and only in Europe. Previous studies have suggested that strains of *A. phagocytophilum* acquire different host tropism [5,27] and pathogenicity [8,28–30]. Up to date, *A. phagocytophilum* with a known pathogenicity for animals and humans have been reported within lineage 1; thus we suggest that the presence of serin at position 242 of groEL may predict a potential to cause disease in humans or domestic animals. The same observation has previously been reported by Petrovec [5].

In our study we found that *A. phagocytophilum* belonging to different *groESL* lineages, with different variants of 16S rRNA genes and with different organization of the *ankA* gene, co-circulated in the same geographical regions in Estonia, Russia and Belarus. We suggest that ticks may harbour all repertoires of *A. phagocytophilum* that are circulating in this region, while genetic variants of *A. phagocytophilum* are segregated in specific natural hosts. Thereby ticks may be an optimal source for studies on the diversity of *A. phagocytophilum* in various geographical regions.

Acknowledgements

The preliminary results of this work were presented at the EDEN International Conference, Montpellier, France, 10–12 May 2010. The sequences detected in the present study were deposited in the GenBank database under numbers from HQ629901 to HQ629932.

Transparency Declaration

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PUBLICATION III

Detection and characterization of *Babesia* Species in *Ixodes* ticks in Estonia

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Detection and Characterization of *Babesia* Species in *Ixodes* Ticks in Estonia

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Abstract

The presence of *Babesia* spp. was studied in 2603 *Ixodes ricinus* and *Ixodes persulcatus* ticks collected at seven sites in Estonia. By reverse line blot screening, *Babesia* spp. was detected in 36 (1.4%) ticks, among them 18 (0.7%) were further recognized by a *Babesia microti* probe, 3 (0.1%) by a *Babesia divergens* probe, and the other 15 (0.6%) were recognized only by the universal *Babesia* spp. "catch all" probe. Sequence analyses of 6 of these 15 samples revealed that all of them belonged to *Babesia* sp. EU1. *B. microti* was detected in both tick species *I. ricinus* and *I. persulcatus* at the seven sites, whereas *B. divergens*-like and *Babesia* sp. EU1 were found only in *I. persulcatus* and *I. ricinus*, respectively. Genetic characterization based on partial 18S rRNA showed that the Estonian sequences of *B. microti*, *B. divergens*-like, and *Babesia* sp. EU1 share a high rate of similarity and are closely related to sequences from other European countries, Siberia, and United States. The present study demonstrated for the first time the existence and distribution of *Babesia* spp. in *I. persulcatus* and *I. ricinus* ticks in Estonia.

Key Words: *Babesia divergens*—*Babesia microti*—*Babesia* species—*B. venatorum*—Estonia—*Ixodes persulcatus*—*Ixodes ricinus*—Prevalence—Ticks.

Introduction

Babesia (Apicomplexa, Piroplasmida) are protozoan parasites transmitted by *Ixodes* ticks to mammals and infecting the host erythrocytes. *Babesia microti* and *Babesia divergens* are the main etiologic agents of human babesiosis in the United States and Europe, respectively (Telford et al. 1993, Brasseur and Gorenflot 1996). In Europe, ~40 human cases in splenectomized or immunocompromised patients due to *B. divergens* and *B. divergens*-like infections have been reported (Vannier and Krause 2009) with up to 42% mortality rate (Gorenflot et al. 1998). Moreover, a new non-*B. divergens* species EU1 (proposed name *Babesia venatorum*) has been recently described as a human pathogen in Italy, Austria, and Germany (Herwaldt et al. 2003, Häselbarth et al. 2007) and also detected in ticks and roe deer (Telford and Goethert 2004, Duh et al. 2005, Casati et al. 2006, Bonnet et al. 2007). The first European human case caused by *B. microti* was reported in Germany (Hildebrandt et al. 2007) and a wide distribution of this pathogen in ticks and small mammals has been demonstrated in Europe (Duh et al. 2001, Skotarczak and Cichocka 2001, Foppa et al. 2002, Goether and Telford 2003, Kalman et al. 2003). In Estonia, no human cases due to *Babesia* infection have been registered. However, human cases have been reported in neighboring countries such as Sweden and Finland (Uhnoo et al. 1992, Haapasalo et al. 2010), and the circulation of *Babesia* in the tick population was reported in Lithuania and Norway (Radzijevskaja et al. 2008).

Ticks from the genus *Ixodes* spp. are the main vectors involved in *Babesia* transmission. In Estonia, two tick species, *I. ricinus* and *I. persulcatus*, are prevalent and widely distributed, whereas in other European countries, *I. ricinus* is the most representative tick and plays a central role in the transmission of *Babesia* spp. The distribution of *I. persulcatus* is restricted to southeastern Estonia, whereas *I. ricinus* circulates in the whole territory of Estonia (Golovljova et al. 2004).

No studies concerning *Babesia* have been performed in Estonia. The present study was undertaken to investigate the prevalence of *Babesia* in ticks collected from different parts of Estonia and to characterize the pathogen by molecular methods.

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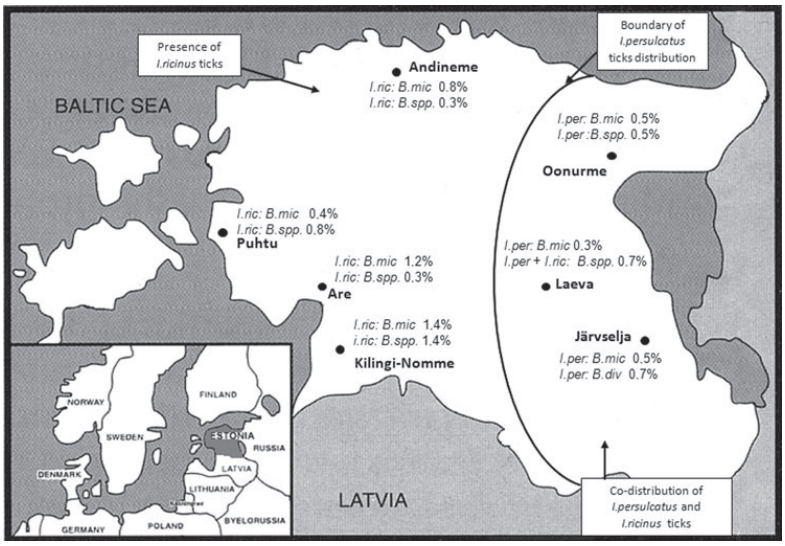

FIG. 1. Tick sampling sites in Estonia (Andineeme, Oonurme, Laeva, Järvselja, Kilingi-Nõmme, Are, Puhtu) and the prevalence of *Babesia* spp. The areas of *Ixodes persulcatus* and *Ixodes ricinus* distribution are indicated.

Materials and Methods

Collection of ticks

Tick sampling was performed from April to November 2006–2008 at different sites in Estonia (Andineeme, Oonurme, Laeva, Järvselja, Kilingi-Nõmme, Are, Puhtu) (Fig. 1). Ticks were collected from the vegetation by flagging and pooled into groups of five adults or nymphs according to tick species, sampling date, and place of collection. Ticks (adults and nymphs) from incomplete pools were processed individually. Pools or individual ticks were washed with sterile phosphate-buffered saline, homogenized in 400 μL of phosphate-buffered saline and stored at -70°C. Two hundred microliters of suspension was used for DNA extraction.

DNA extraction

DNA was extracted with TriPure RNA isolation reagent (Roche Diagnostics) according to the manufacturer's instructions, resuspended in 50 μL of water, and stored at -20°C.

Polymerase chain reaction for the amplification of partial 18S rRNA gene and reverse-line blot hybridization

For screening of ticks, reverse-line blot (RLB) was performed as described earlier (Gubbels et al. 1999). Briefly, the partial 18S rRNA gene of the *Babesia* spp. was amplified with forward primer RLB-F2 and reverse primer RLB-R2 (Table 1). Polymerase chain reaction (PCR) amplification was carried out in a total reaction volume of 50 μL, which contained the following mix of reagents: GeneAmp 10×PCR buffer II, $MgCl_2$ (1 mM), dNTPs (200 μM) (Fermentas, EE), 500 nM of forward and reverse primers, AmpliTaq DNA polymerase (1.25 U; Applied Biosystems, Roche), and 10 μL of target DNA. The PCR conditions consisted of a denaturation step of 3 min at 94°C; followed by 10 cycles of touchdown program, consisting of 20 s of denaturation at 94°C, 30 s at annealing temperature decreased from 67°C to 58°C in each cycle, and an extension step of 30 s at 72°C; and then 40 cycles of denaturing step of 20 s at 94°C, an annealing step of 30 s at 57°C, and an extension step of 30 s at 72°C. A final extension step of 10 min at 72°C completed the program.

Preparation of RLB membrane, hybridization, and subsequent stripping of the membrane were carried out as previously described (Gubbels et al. 1999), with the following modifications. The amplification reaction volume (50 μL) was loaded onto the blot after dilution with 2× SSPE (360mM NaCl, 20 mM $Na_2HPO_4 \times H_2O$, 2 mM EDTA)–0.1% sodium dodecyl sulfate to a total volume of 150 μL. The temperature of the two 10-min posthybridization washes in 2×SSPE–0.5% sodium dodecyl sulfate was increased to 51°C. After developing, the PCR products were stripped from the membrane.

All the nucleotide sequences of the species-specific oligonucleotide probes containing a N-terminal *N*-(trifluoracetamidohexyl-cyanoethyle, *N*,*N*-diisopropyl phoshoramidite)-C6 amino linker were synthesized by TAGC. The sequences of the oligonucleotide probes are shown in Table 1.

Sequencing of the partial 18S rRNA gene

The samples positive by the RLB assay were amplified by nested PCR for sequencing of the partial 18S rRNA gene with outer primers PIRO-A and RLB-R2 and inner primers RLB-F2 and PIRO-B (Table 1). The PCR amplifications were performed in a total volume of 25 μL as follows: GeneAmp 10× PCR buffer II, $MgCl_2$ 1.5 mM for the first PCR and 0.5 mM for the nested PCR, dNTPs 200 μM for the first PCR and 400 μM for the nested PCR, 1 μM of forward and reverse primers, AmpliTaq DNA polymerase (1.25 U; Applied Biosystems,

Table 1. Sequences of Oligonucleotide Probes and Polymerase Chain Reaction Primers Used for the Detection of *Babesia* Spp. in Ticks

Oligonucleotide probes or primers	*Sequences of oligonucleotide probes and PCR primers (5′→3′)*	*References*
Probes		
Babesia spp. ("catch all")	5′amino-TAATGGTTAATAGGARCRGTTG	Oura et al. (2004)
Babesia microti	5′amino-CTTCCGAGCGTTTTTTTATT	Garcia-Sanmartin et al. (2008)
Babesia divergens	5′amino-GTTAATATTGACTAATGTCGA	Gubbels et al. (1999)
Primers for RLB and sequencing		
RLB-F2	GACACAGGGAGGTAGTGACAAG	Georges et al. (2001)
RLB-R2	5′biotin-CTAAGAATTTCACCTCTGACAGT	Georges et al. (2001)
PIRO-A	ATTACCCAATCCTGACACAGGG	Armstrong et al. (1998)
PIRO-B	TTAAATACGAATGCCCCCAAC	Armstrong et al. (1998)

PCR, polymerase chain; RLB, reverse-line blot.

Roche), and 5 μL of target DNA. The cycling conditions were an initial denaturation for 1 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 59°C, and 1 min at 72°C. For the nested PCR, the annealing temperature was 55°C.

The amplified products of the nested PCR were purified with GFX-PCR kit (Amersham Biosciences) according to the manufacturer's instructions. The purified PCR products were used as templates in the sequencing reaction using the dideoxynucleotide chain termination method with BigDye Terminator Cycle Sequencing Ready Reagent Kit (ABI PRISM, PE Applied Biosystems). The ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) was used for sequence analysis. The obtained sequences were edited using the BioEdit program (www.mbio.ncsu.edu/BioEdit/bioedit.html).

Phylogenetic analysis

Phylogenetic analysis was carried out with the TREE-PUZZLE 5.2 version package. For phylogenetic tree reconstruction, a quartet puzzling Maximum Likelihood method was used and 10,000 puzzling steps were applied using the Hasegawa-Kishino-Yano model for substitution (Hasegawa et al. 1985). The sequences of the partial 18S rRNA genes for the *Babesia* species were retrieved from the GenBank database and aligned by BioEdit program (www.mbio.ncsu.edu/BioEdit/bioedit.html). A *Plasmodium falciparum* 18S rRNA sequence was included as outgroup.

Nucleotide sequence accession numbers

The sequences of the partial 18S rRNA gene detected in the present study were deposited in the GenBank database under numbers running from HQ629933 to HQ629948.

Results

Detection of Babesia *sp. in ticks by RLB hybridization*

A total of 2603 ticks including 938 *I. persulcatus* (577 adults and 361 nymphs) and 1665 *I. ricinus* (587 adults and 1078 nymphs) were collected from different parts of Estonia in 2006–2008 (Fig. 1).

All ticks were tested for the presence of *Babesia* spp. by RLB. The presence of *Babesia* was detected in 36 pools of ticks (1.4%), among them 18 (0.7%) were recognized by the *B. microti* probe, 3 (0.1%) by *B. divergens* probe, and the other 15 (0.6%) were recognized only by the universal *Babesia* spp. "catch all" probe on the membrane (Table 2). *B. microti* was detected in 4 pools of *I. persulcatus* (3 pools of females and 1 pool of males) from the

Table 2. *Babesia* Sp. Detection in Ticks by Reverse-Line Blot Hybridization

Sampling sites	*Species of tick*	B. microti *No. of infected samples/no. of investigated samples (MIR %)*	B. divergens *No. of infected samples/no. of investigated samples (MIR %)*	Babesia *sp. No. of infected samples/no. of investigated samples (MIR %)*	*Total No. of infected samples/no. of investigated samples (MIR %)*
Puhtu	*Ixodes ricinus*	2/475 (0.4%)	0/475	4/475 (0.8%) (2 EU1)[a]	6/475 (1.3%)
Are		4/321 (1.2%)	0/321	1/321 (0.3%)	5/321 (1.5%)
Andineme		3/359 (0.8%)	0/359	1/359 (0.3%) (1 EU1)[a]	4/359 (1.1%)
Kilingi-Nõmme		5/345 (1.4%)	0/345	5/345 (1.4%) (1 EU1)[a]	10/345 (2.9%)
Oonurme	*Ixodes persulcatus*	1/209 (0.5%)	0/209	1/209 (0.5%)	2/240 (0.8%)
	I. ricinus	0/31	0/31	0/31	
Laeva	*I. persulcatus*	1/320 (0.3%)	0/320	1/320 (0.3%)	4/417 (0.9%)
	I. ricinus	0/97	0/97	2/97 (2%) (2 EU1)[a]	
Järvselja	*I. persulcatus*	2/409 (0.5%)	3/409 (0.7%)	0/409	5/446 (1.1%)
	I. ricinus	0/37	0/37	0/37	
Total		18/2603 (0.7%)	3/2603 (0.1%)	15/2603 (0.6%) (6 EU1)[a]	36/2603 (1.4%)

[a]No. of samples confirmed as EU1 by sequencing.
MIR, minimum infection rate.

eastern part of Estonia (Laeva, Oonurme and Järvselja) and in 14 *I. ricinus* nymph pools, which were collected in the western part of Estonia (Andineeme, Puhtu, Are, and Kilingi-Nõmme). Three pools—*I. persulcatus* (two males and one female) from the Järvselja collection site—reacted with the *B. divergens* probe. Fifteen tick pools (13 pools of *I. ricinus* nymphs and 2 of *I. persulcatus*) with undetermined *Babesia* species by RLB originated from Oonurme, Puhtu, Laeva, Andineeme, Kilingi-Nõmme, and Are. The minimum infection rate was calculated by assuming that only one tick out of five in a pool was positive. The prevalence rates of *Babesia* species at different geographical sites are shown in Figure 1 and Table 2.

Sequencing and phylogenetic analysis of partial 18S rRNA gene

For confirmation of the *Babesia* findings, 16 samples found to be positive by RLB (8 recognized by the *B. microti* probe, 2 recognized by the *B. divergens* probe, and 6 recognized by the *Babesia* spp. "catch all" probe) were amplified and sequenced in the 18S rRNA gene region.

B. microti DNA was amplified from *I. persulcatus* (Est603, Est884) and *I. ricinus* (Est 923, Est939, Est941, Est1033, Est1078, Est1098). Seven of eight amplicons were found identical for the 378-bp amplicon of the 18S rRNA gene to sequences previously found in a human blood sample (GenBank EF413181) in Germany and also to sequences from *I. ricinus* ticks and rodents from Slovenia (GenBank AF373332, AY149572) and Switzerland (GenBank AF494286). However, the sequence of the *B. microti* sample Est884 differed from the other Estonian sequences by two nucleotide substitutions at positions 614 (A/G) and 699 (T/C), which made it distinct from other published sequences by at least one substitution. On the phylogenetic tree, the Estonian *B. microti* samples clustered together with strains belonging to the zoonotic "US" type, which is distributed worldwide, and has been reported as pathogenic for humans (Gray et al. 2010) (Fig. 2). The Estonian sequences share a high rate of similarity (99.7%–100%) with other sequences belonging to the "US" type; 98.4% similarity was found with sequences belonging to the Hobetsu and Kobe types, whereas the lowest similarity rate (95.8%–96.3%) was demonstrated with Munich-type strains.

The two *B. divergens*-like samples (Est622 and Est746) were amplified from *I. persulcatus* ticks and generated 350-bp amplicons of the partial 18S rRNA gene. They were identical to sequences previously detected in roe deer and reported as *B. divergens* in Slovenia (GenBank AY572456), Poland (GenBank DQ083544), and Spain (GenBank DQ866844), whereas identical sequences were classified as *Babesia capreoli* in France (GenBank AY726009, FJ944827, FJ944828). Therefore, the distinction between the two closely related *Babesia* species, *B. divergens* and *B. capreoli*, remains questionable. We reported our Estonian sequences as *B. divergens*-like, although the Estonian samples had G at nucleotide position 631 and T at nucleotide position 663 of the 18S rRNA gene as that was proposed as a feature of *B. capreoli* (Malandrin et al. 2010). Other *B. divergens* and *B. divergens*-like sequences from Europe, United States, and West Siberia showed 98.2%–99.7% of identity with the Estonian samples amplified in the present study (Fig. 2).

The 6 sequenced samples (Est788, Est670, Est916, Est1148, Est1362, and Est1444) of the 15 ones found to be positive by RLB only with the universal *Babesia* spp. probe were identified as EU1 after sequencing. All six were amplified from *I. ricinus* ticks and produced 350-bp amplicons that were identical to the *Babesia* sp. EU1 sequences detected from *I. ricinus* in France (GenBank FJ215873) and Slovenia (GenBank AY553915), from roe deer in Slovenia (GenBank AY572457) and France (GenBank EF185818), from human blood in Italy (GenBank AY046575), and from *I. persulcatus* in West Siberia (GenBank GU734773) (Fig. 2). *Babesia* sp. EU1 was found only in *I. ricinus* even in an area (Laeva) where the proportion of *I. ricinus* ticks is only 20%.

Discussion

The present study revealed for the first time the presence of *Babesia* in Estonia. Our data showed that the potentially pathogenic species *B. microti*, *Babesia* sp. EU1, and *B. divergens*-like

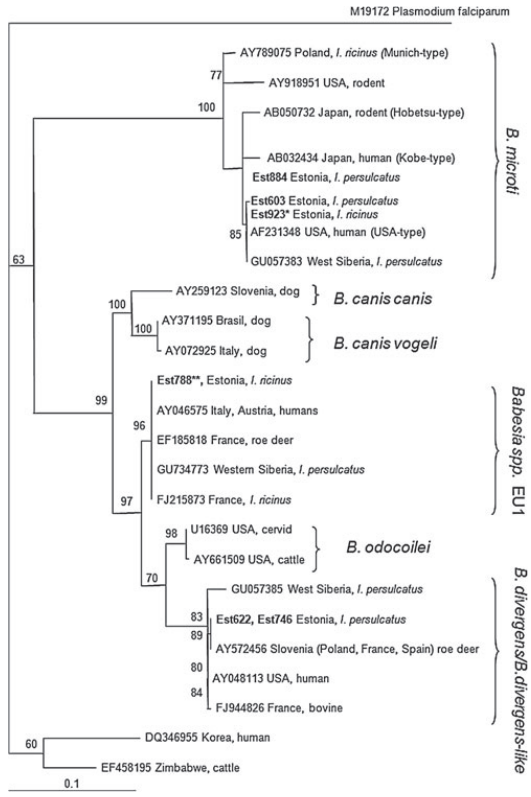

FIG. 2. Phylogenetic tree based on partial 18S rRNA gene (378 bp) sequences of *Babesia* species. The phylogenetic tree was constructed using the quartet puzzling maximum likelihood method (TREE-PUZZLE program). The numbers on the nodes are bootstrap values based on 10,000 replicates (only values >70% are shown). *Plasmodium falciparum* has been used as the outgroup. The GenBank accession numbers for the sequences used in the present study are given. Est923* is identical to sequences Est939, Est941, Est1033, Est1078, and Est1098; Est788** is identical to sequences Est670, Est916, Est1148, Est1362, and Est1444. The Estonian sequences from this study are in bold.

circulated in *I. ricinus* and *I. persulcatus* ticks in different areas in Estonia, with a total prevalence rate of 1.4%. A similar prevalence in ticks has been reported from South Germany (1%) (Hartelt et al. 2004) and Switzerland (0.7%–1.7%) (Casati et al. 2006). However, much higher *Babesia* spp. prevalence rates were reported in *I. ricinus* ticks from Slovenia (9.6%) (Duh et al. 2001), France (6.1%–20.6%) (Halos et al. 2005, Cotté et al. 2010), and Austria (51.8%) (Blaschitz et al. 2008). The distribution of infected ticks is mosaic and varies during seasons and years; thus, a comparison of tick-borne pathogen prevalence rates between countries should be performed with caution.

B. microti was the most frequently found *Babesia* species in Estonia. We found identical *B. microti* sequences in *I. persulcatus* and *I. ricinus* ticks; thus, we suggest that both tick species play a role in the transmission and maintenance of *B. microti* in natural foci in Estonia. All detected sequences clustered together with zoonotic "US"-type *B. microti* strains, which were widely distributed in Europe, United States, and West Siberia. The possibility to utilize several *Ixodes* species as vector may be the reason for worldwide spreading of *B. microti* "US"-type strains.

We found *B. divergens*-like (probably *B. capreoli*) sequences only in *I. persulcatus* ticks, whereas in the other European countries such as Slovenia, Germany, and Switzerland, this species of *Babesia* was detected in *I. ricinus* (Duh et al. 2001, Hartelt et al. 2004, Casati et al. 2006). Moreover, the Estonian strains were identical to the other European strains in the partial 18S rRNA gene region; thus, we suggest that *B. divergens* could use both tick vectors for its transmission and maintenance in natural foci (Fig. 2).

In the present study, *Babesia* sp. EU1 was detected only in *I. ricinus* ticks even in areas where *I. persulcatus* predominated (85% of collected ticks). Recently, it was demonstrated that *I. ricinus* is a competent vector for *Babesia* sp. EU1 (Bonnet et al. 2007, 2009, Becker et al. 2009). However, *Babesia* sp. EU1 was found also in *I. persulcatus*, suggesting it as a vector in West Siberia (Rar et al. 2010). Our Estonian sequences were identical to the European as well as Siberian ones in the partial 18S rRNA gene region (Fig. 2). Thus, it needs to be clarified whether *Babesia* sp. EU1 is not able to utilize *I. persulcatus* as a vector in Estonia or the detection of this *Babesia* species only in *I. ricinus* is due to a collection bias.

A close relationship of the Estonian and other *Babesia* sequences was confirmed by phylogenetic analysis. On the phylogenetic tree based on the partial 18S rRNA gene, the Estonian sequences clustered together with other *B. microti*, *B. divergens*/*B. divergens*-like, and *Babesia* sp. EU1 sequences from Europe, United States, and West Siberia with a high bootstrap support (Fig. 2). Moreover, the analyzed sequences of *B. microti* and *Babesia* sp. EU1 were identical in the 18S partial gene region to sequences reported in ill humans, although until now there have been no reports of human infection due to *Babesia* spp. in Estonia.

The results of the present study demonstrated for the first time the presence of *B. microti*, *B. divergens*-like, and *Babesia* sp. EU1 in natural populations of *Ixodes* ticks in Estonia, which should be considered as a possible risk of transmission to humans.

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Disclaimer

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Disclosure Statement

No competing financial interests exist.

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