Tick-borne encephalitis (TBE) is a vector-borne disease with severe neurological outcomes. The causative agent, TBE virus (TBEV) is usually transmitted to humans through tick-bite. Environmental factors play an important role in TBE distribution by regulating interactions between ticks and humans. Climate change has contributed to increased tick abundance and incidence of tick-borne diseases, and between 10,000 and 15,000 human TBE cases are reported annually in Europe and Asia. Naveed Asghar has studied the probability of seasonal synchrony of questing ticks and total tick abundance, which are important factors for persistence of TBEV in nature. He has identified TBEV quasispecies within the virus pools from cell culture, infected mice brain, questing- and blood-feeding ticks. His data indicate that the selection pressure faced by the TBEV quasispecies in different hosts affects the virus quasispecies evolution within respective host. He has also investigated the putative role of quasispecies and the 3’ non-coding region in TBEV virulence. In addition, he identified similarities between the TBEV quasispecies pools of two TBEV strains from a natural focus, indicating TBEV persistence in nature.

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Ticks and Tick-borne Encephalitis Virus
Ticks and Tick-borne Encephalitis Virus
From nature to infection

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Vector-borne diseases are an increasing global threat to humans due to climate changes, elevating the risk of infections transmitted by mosquitoes, ticks, and other arthropod vectors. *Ixodes ricinus*, a common tick in Europe, transmits dangerous tick-borne pathogens to humans. Tick-borne encephalitis (TBE) is a vector-borne disease caused by TBE virus (TBEV). Climate change has contributed to increased tick abundance and incidence of tick-borne diseases, and between 10,000 and 15,000 human TBE cases are reported annually in Europe and Asia. TBEV shows a patchy geographical distribution pattern where each patch represents a natural focus. In nature, TBEV is maintained within the tick-rodent enzootic cycle. Co-feeding is the main route for TBEV transmission from infected to uninfected ticks and for maintenance within the natural foci. The increasing number of TBE cases in Scandinavia highlights the importance of characterizing additional TBEV sequences and of identifying novel natural foci, and in this work we sequenced and phylogenetically characterized four TBEV strains: Saringe-2009 (from a blood-fed nymph), JP-296 (from a questing adult male), JP-554 (from a questing adult male), and Mandal-2009 (from a pool of questing nymphs, n = 10). Mandal-2009 represents a TBEV genome from a natural focus in southern Norway. Saringe-2009 is from a natural endemic focus in northern Stockholm, Sweden, and JP-296 and JP-554 originate from a natural focus “Torö” in southern Stockholm. In addition, we have studied the effect of different biotic and abiotic factors on population dynamics of *I. ricinus* in southern Stockholm and observed significant spatiotemporal variations in tick activity patterns. Seasonal synchrony of immature stages and total tick abundance are important factors for the probability of horizontal transmission of TBEV among co-feeding ticks. We found that the probability of co-occurrence of larvae, nymphs, and female adults was highest during early summer whereas increasing vegetation height and increasing amounts of forest and open water around the study sites had a significant negative effect on co-occurrence of larvae, nymphs, and female adults.

The proximal part of the 3´non-coding region (3´NCR) of TBEV contains an internal poly(A) tract, and genomic analysis of Saringe-2009 revealed variability in the poly(A) tract indicating the existence of different variants within the TBEV pool of Saringe-2009. Like other RNA viruses, TBEV exists as swarms of unique variants called quasispecies. Because Saringe-2009 came from an engorged nymph that had been feeding on blood for >60 h, we propose that Saringe-2009 represents a putative shift in the TBEV pool when the virus switches from ectothermic/tick to endothermic/mammalian environments. We investigated the role of poly(A) tract variability in replication and virulence of TBEV by generating two infectious clones of the TBEV strain Toro-2003, one with a short/wild-type (A)_3C(A)_6 poly(A) tract and one with a long (A)_3C(A)_38 poly(A) tract. The infectious clone with the long poly(A) tract showed poor
replication in cell culture but was more virulent in C57BL/6 mice than the wild-type clone. RNA folding predictions of the TBEV genomes suggested that insertion of a long poly(A) tract abolishes a stem loop structure at the beginning of the 3´NCR. Next generation sequencing (NGS) analysis of the TBEV genomes after passaging in cell culture and/or mouse brain revealed molecular determinants and quasispecies structure that might contribute to the observed differences in virulence. Our findings suggest that the long poly(A) tract imparts instability to the TBEV genome resulting in higher quasispecies diversity that in turn contributes to TBEV virulence. Phylogenetic analysis of Saringe-2009, JP-296, JP-554, and Mandal-2009 predicted a strong evolutionary relationship among the four strains. They clustered with Toro-2003, the first TBEV strain from Torö, demonstrating a Scandinavian clade. Except for the proximal part of the 3´NCR, TBEV is highly conserved in its genomic structure. Genomic analysis revealed that Mandal-2009 contains a truncated 3´NCR similar to the highly virulent strain Hypr, whereas JP-296 and JP-554 have a genomic organization identical to Toro-2003, the prototypic TBEV strain from the same natural focus. NGS revealed significantly higher quasispecies diversity for JP-296 and JP-554 compared to Mandal-2009. In addition, single nucleotide polymorphism (SNP) analysis showed that 40% of the SNPs were common between quasispecies populations of JP-296 and JP-554, indicating the persistence and maintenance of TBEV quasispecies within the natural focus.

Taken together, these findings indicate the importance of environmental factors for the occurrence pattern of the different life-stages of the tick vector, which are important for the persistence of TBEV in nature. Our findings also show that the selection pressure exerted by specific host also affects the population structure of the TBEV quasispecies. In addition, our results further demonstrate that the evolution of quasispecies has effect on TBEV virulence in mice.

**Keywords:** Co-occurrence, Environmental factors, Flavivirus, *Ixodes ricinus*, Natural foci, Non-coding region, Poly(A) tract, Quasispecies, Questing ticks, Scandinavia, Tick-borne encephalitis virus, Vegetation.
Sammanfattning


Förutom den genetiska sekvenseringen av TBEV har vi också studerat effekten av olika biotiska och abiotiska faktorer på populationsdynamik av *I. ricinus* i södra Stockholm och observerade variation i fästingsaktivitetsmönster både temporalt och spatialt. Förekomstmönster av fästinglarver, nymfer och vuxna honor, och det totala antalet fästingar är viktiga faktorer för sannolikheten för horisontell överföring av TBEV mellan fästingar. Vi fann att sannolikheten för synkron förekomst av larver, nymfer och honor var högst under försommaren. Vegetationshöjd, mängden skog och mängd öppet vatten runt undersökningsområden hade signifikanta negativa effekter på sannolikheten för att larver, nymfer och honor skulle förekomma samtidigt.

Den variabla delen av den icke-kodande 3´regionen (3´NCR) av TBEV-genomet innehåller ofta en intern poly(A) sekvens. Liksam andra RNA virus,
förekommer TBEV som så kallade ”quasispecies” vilka definieras som grupper av olika genetiska varianter av virus. Genomanalysen av TBEV-stam Saringe-2009 avslöjade variation i poly(A) sekvensen vilket indikerar förekomst av ”quasispecies”. Eftersom Saringe-2009 kom från en blodfylld nymf som hade sugit blod i > 60 timmar, föreslår vi att Saringe-2009 visar en förändring i ”quasispecies”-poolen när viruset överförs från extoterm fästingmiljö till endoterm däggdjurens miljö. Vi undersökte poly(A)-sekvensens variabilitet och dess roll vid replikering och för virulens hos TBEV, genom att skapa två infektiösa kloner av Torö-2003 stammen; en med en kort/vild-typ (A)₃C(A)₆ poly(A) sekvens, och en med en lång (A)₃C(A)₃₈ poly(A)-sekvens. Den infektiösa klonen med lång poly(A)-sekvens replikerade sämre än vildtypklonen i cellkultur, men (A)₃C(A)₃₈ poly(A) var mer virulent i C57BL/6-möss än (A)₃C(A)₆ poly(A). Datosimulering av TBEV genomets sekundär RNA-struktur visade att de längre poly(A)-sekvensererna påverkar veckningen av en specifik sekundärstruktur (SL14) i början av 3´NCR. Djupsekvenseringsanalys av TBEV genom avslöjade skillnader för specifik gener och ”quasispecies”-strukturen efter passering i cellkultur och/eller mushjärna. Dessa förändringar föreslås bidra till de observerade skillnaderna i virulens. Våra resultat indikerar att den långa poly(A) sekvensen ger instabilitet i TBEV genomet, vilket resulterar i ökad mångfald av ”quasispecies”-populationen som i sin tur kan bidra till TBEV-virulens.


Sammantaget visar dessa studier att miljöfaktorer påverkar förekomsten av fästingvektorn och dess olika livsstadien, vilket är en bakomliggande faktor för utbredning av TBEV i naturliga fokusområden. De visar även på att vårdmiljön påverkar strukturen för ”quasispecies”-populationen. Dessutom visar våra studier att evolution och utveckling av ”quasispecies”-strukturen kan påverka virulensen för TBEV i möss.
“With faith, discipline and selfless devotion to duty, there is nothing worthwhile that you cannot achieve.”

Jinnah

Dedicated to my beloved parents, siblings, wife & Jamal Ahmed
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Publications

This thesis is based on following articles


Submitted


Submitted


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My Contributions

My contributions to the respective articles are as follows.

I. I conceived, designed, and performed the experiments; analysed the data; and wrote the manuscript.

II. I designed the experiments, performed the molecular cloning and reverse genetics work, performed the next generation sequencing, \textit{in silico} RNA folding simulations, and wrote the manuscript. I also contributed to data analysis and manuscript review.

III. I designed and conducted the experiments, analysed the data, and wrote the manuscript.

IV. I performed the tick sampling and wrote the manuscript. I also contributed to study design, data analysis, and reviewing the manuscript.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>C</td>
<td>Capsid</td>
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<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>CS</td>
<td>Cyclization sequence</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>E</td>
<td>Envelope</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>Eu-TBEV</td>
<td>European TBEV</td>
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<tr>
<td>FE-TBEV</td>
<td>Far eastern TBEV</td>
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<tr>
<td>GAGs</td>
<td>Glycoaminoglycans</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>NCR</td>
<td>Non-coding region</td>
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<tr>
<td>NGS</td>
<td>Next generation sequencing</td>
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<td>NS</td>
<td>Non-structural</td>
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<tr>
<td>nt</td>
<td>Nucleotide</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>pfu</td>
<td>Plaque-forming unit</td>
</tr>
<tr>
<td>prM</td>
<td>Precursor membrane</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse-transcription PCR</td>
</tr>
<tr>
<td>RC</td>
<td>Replication complex</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>Sib-TBEV</td>
<td>Siberian TBEV</td>
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<tr>
<td>SL</td>
<td>Stem loop</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single-stranded RNA</td>
</tr>
<tr>
<td>sfRNA</td>
<td>Subgenomic flavivirus RNA</td>
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<tr>
<td>TBE</td>
<td>Tick-borne encephalitis</td>
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<td>TBEV</td>
<td>Tick-borne encephalitis virus</td>
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<tr>
<td>TBPBs</td>
<td>Tick-borne pathogens</td>
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<tr>
<td>YFV</td>
<td>Yellow fever virus</td>
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Vector-borne diseases are an increasing global threat to humans and livestock because climate changes have elevated the risk of infections transmitted by arthropod vectors such as ticks and mosquitoes. Tick-borne encephalitis (TBE) is a vector-borne disease resulting in severe neurological symptoms and potentially permanent brain damage (Holzmann, 2003). The causative agent – TBE virus (TBEV) – is usually transmitted to humans through tick bites (Zilber, 1939). *Ixodes ricinus* represents the dominant tick in Europe that transmits dangerous tick-borne pathogens (TBPs) such as *Anaplasma phagocytophilum*, *Rickettsia helvetica*, *Borrelia burgdorferi* s.l., and TBEV to humans (de la Fuente *et al.*, 2008; Gray, 2002; Randolph, 2009). TBE is an increasing health burden, with 10,000 to 15,000 cases reported annually in Europe and Asia (Dobler, 2010).

Environmental factors play an important role in TBE distribution by regulating interactions between ticks and humans. Climate change resulting in a warmer climate will facilitate tick-human interactions by influencing human behaviour, speeding up development of the tick vector, and prolonging the vegetation growing period to provide better habitats both for ticks and host animals resulting in increased tick abundance and broader distribution limits (Jaenson & Lindgren, 2011). Climate is described as the main restricting factor at the northern distribution limit of *I. ricinus* in Sweden (Jaenson *et al.*, 1994), and expansion of the northern distribution limit of *I. ricinus* has been observed in Sweden over the last few decades (Jaenson *et al.*, 2012a). In addition, increased *I. ricinus* abundance and disease incidence associated with climate changes, i.e. warm, humid summers and mild winters, have also been reported (Bennet *et al.*, 2006; Talleklint & Jaenson, 1998). TBE incidence coincides with seasonal tick activity patterns, and it has been claimed that the northwards movement of TBE disease is associated with an increase in temperature (Daniel *et al.*, 2009; Zeman & Benes, 2004), which corresponds to increased tick activity at higher altitudes (Daniel *et al.*, 2009).

Climate likely regulates the distribution patterns of ticks and tick-borne diseases on a large scale. However, an understanding of small-scale variations in landscape characteristics and their implications for ticks and human pathogens like TBEV is also required. The abundance of small mammals and ticks depends to a large extent on local land cover (Perez *et al.*, 2016). In two earlier studies, the authors showed positive relationships between forest cover and tick abundance (Del Fabbro *et al.*, 2015; Perez *et al.*, 2016). Forest cover also showed a significant positive relationship with wood mice, but had a negative or non-significant
effect on bank voles. There is still a lack of general understanding of how local environmental patterns affect tick distribution and the risk of humans becoming infected with TBEV and other TBPs.

TBEV is maintained in so-called natural foci, where it circulates between ticks and vertebrate hosts under defined ecological conditions (Estrada-Pena & de la Fuente, 2014; Suss, 2011). These natural foci have also been proposed to develop due to the seasonal synchrony between the questing activity of tick larvae and nymphs (Labuda & Randolph, 1999; Randolph, 2000). However, additional studies comparing questing activity of ticks at natural TBEV foci and in areas without TBEV are required to determine if synchrony in the questing activity of larvae and nymphs is responsible for TBEV persistence in its natural foci. Accurate, representative sampling of all of the questing life cycle stages of the tick is required to perform this type of research. These studies involve tick collection from trapped animals or tick sampling using a cotton blanket in the field without trapping host animals. Several techniques have been developed to improve tick sampling (Dobson et al., 2011; Melik et al., 2007; Soleng & Kjelland, 2013; Tack et al., 2011), but an efficient technique for representative sampling of all the questing lifecycle stages is still missing.

The first TBE infection was reported in Sweden in 1954 (Holmgren & Forsgren, 1990), and the number of TBE cases in Sweden is now about 200 to 300 cases per year (Jaenson et al., 2012b). In 1997, the first TBE case was described in Norway (Skarpaas et al., 2006). Although a significant number of TBEV genomes are available, our understanding of the emergence of TBEV in Norway is very poor. Most of the TBEV genomes available in the database have been sequenced after propagation in cell culture, chicken egg, or mouse brain. However, for a better understanding of TBEV evolution in nature and its spread e.g. into Norway, additional TBEV genomic sequences are required from different natural foci in Scandinavia.

TBE has been known for some time, but we still do not have a complete understanding of the pathophysiological mechanism of the disease, although several molecular determinants of TBEV virulence have been demonstrated (Best et al., 2005; Sakai et al., 2015; Sakai et al., 2014; Werme et al., 2008). TBEV exists as a population of distinct variants called quasispecies (Asghar et al., 2014; Enquist & Racaniello, 2013; Luat le et al., 2014), and tick to mammal transmission of TBEV is believed to be responsible for altering the genotype and phenotype of TBEV (Kaluzova et al., 1994; Romanova et al., 2007). This notion is supported by the observation that TBEV virulence in mice is reduced after passaging the virus in I. ricinus (Labuda et al., 1994). However, the virulence reverted to normal after repeated cycles of amplification in mammalian hosts (Kaluzova et al., 1994). A positive relation between quasispecies diversity and the neurotropism of polio and yellow fever virus (YFV) has also been demonstrated (Beck et al., 2014; Vignuzzi et al., 2006), but additional studies exploring the virulence determinants of TBEV
and the role of quasispecies in the pathogenicity of the disease are required. The use of modern DNA sequencing technology allows detailed analysis of viral quasispecies so as to better understand their role in TBE pathogenicity and the persistence of TBEV in nature.
Aims

Paper I
TBEV was first described in Norway in 1997 despite the fact that the virus has been reported in Sweden since 1954. This study aimed to analyse the evolution of Scandinavian TBEV strains. We aimed to sequence the complete genome of the first Norwegian TBEV strain, Mandal-2009, and a Swedish strain, Saringe-2009, to compare genomic differences between the TBEV strains present within questing and blood-feeding ticks.

Paper II
TBE is a disease with severe neurological outcomes, but there is still no clear understanding of the underlying mechanism of TBE pathogenicity. This study aimed to determine the role of the poly(A) tract in genomic stability, replication, and virulence of TBEV. In addition, we studied TBEV evolution in different cellular environments and explored the population structure of TBEV quasispecies responsible for the observed difference in replication and virulence of TBEV.

Paper III
Natural foci of TBEV are known to exist throughout Europe and Asia. However, little is known about the persistence of TBEV within natural foci. The aim of this study was to sequence and phylogenetically characterize two additional TBEV strains – JP-296 and JP-2554 – from TBEV-positive questing ticks sampled at a natural focus in Torö in southern Stockholm, Sweden. In addition, we compared the TBEV populations within questing ticks sampled from natural foci in Sweden and Norway.

Paper IV
Synchronized seasonal activity of larvae and nymphs is required in an established natural TBEV focus, and studies investigating such synchronizations require accurate, representative sampling of all of the questing stages. We established a standardized technique for accurate representative sampling of all the questing stages of *I. ricinus*. Our aim was to analyse if overlapping distributions of larvae, nymphs, and female ticks were responsible for TBE prevalence by comparing controls (ticks from sites with no TBE) to cases (sites with TBE).
Background

1. The disease

1.1 Tick-borne encephalitis
TBE presents with a diversity of symptoms ranging from mild flu-like symptoms to severe encephalitis, which often leads to long-lasting sequelae or permanent brain damage (Holzmann, 2003). The infection was first described by an Austrian physician in 1931 (Schneider, 1931), and in 1939 it was demonstrated that TBEV transmission to humans was through the tick *Ixodes persulcatus* (Zilber, 1939). In addition to tick bites, consumption of unpasteurized milk or dairy products can also cause TBE in humans (Balogh *et al.*, 2010; Hudopisk *et al.*, 2013; Markovinovic *et al.*, 2016). Based on their genomic sequences, TBEV can be classified into three subtypes: European (Eu), Siberian (Sib), and Far Eastern (FE). Although the TBEV subtypes share >93% identity at the amino acid level and exhibit closely related antigenicity, they present with different clinical manifestations. Sib-TBEV and FE-TBEV usually present with monophasic illness, whereas in severe Eu-TBE cases the infection takes a biphasic course after an incubation period of 1–2 weeks. The first phase is characterized by headache, fever, malaise, and muscle pains followed a symptom-free period of ~7 days. During the second phase, about one fourth of the patients develop neurological symptoms (Charrel *et al.*, 2004). Eu-TBEV causes infections with a case fatality rate of 1–2%, whereas more severe disease are caused by Sib-TBEV and FE-TBEV with case fatality rates of 6–8% and 20–40%, respectively (Mandl, 2005).

1.2 Epidemiology of TBE
TBE is an emerging infectious disease in Europe and Asia with 10,000 to 15,000 annually reported cases from the two continents (Dobler, 2010). In Europe, TBE represents the most important viral disease transmitted by the tick vector (Suss, 2011). Over the recent decades, marked increase in TBE prevalence and incidence has been reported in Scandinavia (Jaenson *et al.*, 2012b; Mansfield *et al.*, 2009). The geographical distribution of TBEV subtypes corresponds to the geographical ranges of their respective vectors (Figure 1). TBE is endemic in specific regions of 27 European countries and 6 Asian countries (Suss, 2011), and all three TBEV subtypes have been reported to co-circulate in the three Baltic states (Golovljova *et al.*, 2004).
Traditionally, the epidemiological data of TBEV is inferred from the number of registered TBE cases in an area. However, methods involving TBEV detection in ticks or TBEV antibodies in sera of domestic or wild animals or in animal milk may be used as sentinels for epidemiological studies. Besides environmental factors that control the survival and development of ticks and TBP s, the epidemiology of TBE is also driven by socio-demographic factors and human activities resulting in landscape changes. All of these factors influence the interactions among vectors, pathogens, and hosts (Patz et al., 2003). Over the last few years, TBEV has been identified at higher altitudes and in areas that were previously non-endemic (Suss, 2011). For instance, TBE cases have been reported in Denmark outside Bornholm Island (Fomsgaard et al., 2009), in southern Switzerland (Rieille et al., 2014), and in the mountain regions of Austria (Heinz et al., 2015; Holzmann et al., 2009). In addition, re-emergence of TBEV in north-eastern Germany has also been reported (Frimmel et al., 2014). Sib-TBEV has been identified in western Finland from a TBEV-endemic focus in the Kokkola archipelago of the Baltic Sea indicating a northwest extension of the previously known range of I. persulcatus and Sib-TBEV (Jaaskelainen et al., 2010). In addition, I. persulcatus has recently been identified in northern Sweden, thus enables putative existence of more severe TBEV subtypes in those parts of Sweden. In the Netherlands, the first autochthonous human TBE case was diagnosed in July 2016 (de Graaf et al., 2016).

1.3 TBE natural foci

TBE presents with a patchy geographical distribution pattern where each patch corresponds to a single natural focus that might range from a couple of square meters to several square kilometers (Dobler, 2010; Suss, 2003). Endemic natural
Background

Foci are known to exist throughout Europe and Asia. Natural foci are quite dynamic, and new foci are constantly emerging in both continents (Suss, 2011). Climate-associated transitions of TBE foci from lowlands to submountainous areas have been observed over a 20-year period (Lukan et al., 2010). In addition, an established focus might enter a state of latency and re-emerge after a certain period (Frimmel et al., 2014; Rieille et al., 2014). There is uncertainty regarding the evolution of new TBEV foci, and several theories describing the role of environmental factors, large ungulates, birds, or rodents in the spread of TBEV foci have been proposed (Estrada-Peña & de la Fuente, 2014; Jaenson et al., 2012a). Defining TBEV foci based merely on human TBE cases is unreliable because certain factors like vaccine coverage can make it hard to distinguish between endemic and non-endemic areas (Imhoff et al., 2015; Suss, 2003). Thus in addition to human TBE cases, questing and blood-feeding ticks from humans, birds, and animals as well as tissues and blood samples of field-collected and domestics mammals are used as golden standards to define natural TBEV foci (Imhoff et al., 2015).

2. The vector

2.1 Ticks

Ticks are ectoparasites that ingest blood from a wide host range, including mammals, birds, and reptiles (Jaenson et al., 1994). They belong to the class Arachnida of phylum Arthropoda and are placed in subclass Acari together with mites (Evans, 1992). Among all blood-sucking arthropods, ticks rank first in terms of diversity of pathogens and second to mosquitoes in terms of disease burden transmitted to humans and livestock (Karbowiak et al., 2015; Sonenshine et al., 2002). There are about 900 tick species, and most of them are divided into the soft ticks (Argasidae) and the hard ticks (Ixodidae) (Guglielmone et al., 2010). *I. ricinus* and *I. persulcatus* are hard ticks and represent the dominant tick vectors in Europe and Asia, respectively, and transmit both human and veterinary diseases (de la Fuente et al., 2008). *Ixodes* ticks have no eyes, but they are equipped with sensory organs at the front pair of legs that help them to sense carbon dioxide, heat radiation, odours, and movements (Leonovich, 2004). *Ixodes* ticks transmit diverse TBP, including viruses, bacteria, protozoa, and fungi through their saliva while they feed on their hosts. The feeding time for *Ixodes* ticks ranges from 2–15 days and depends on several factors including, but not limited to, tick species, life stage, attachment site, and host type (Kaufman, 2007; Parola & Raoult, 2001). An adult blood-feeding *Ixodes* female can suck about 200–300 times its unfed bodyweight. While sucking the blood, the female secretes hypoosmotic saliva into the host’s circulation thus concentrating the blood meal to achieve a final engorgement of 100–120 times its body weight (Kaufman, 2010; Parola & Raoult, 2001).
2.2 Tick life cycle

The average *I. ricinus* life cycle is 3 years, but it can reach up to 6 years depending on the climate and host abundance (Parola & Raoult, 2001; Suss, 2003). Four life cycle stages – egg, larva, nymph, and adult – constitute the three-host life cycle of *I. ricinus* (Figure 2). When the questing *I. ricinus* finds a host, it feeds for several days (once per life cycle stage) (Suss, 2003). The larvae and nymphs usually feed on small animals like rodents, whereas adults feed on larger animals like deer, foxes, or wild boars. *I. ricinus* females lay 1000–5000 eggs that hatch into six-legged larvae. After a blood meal, the larvae metamorphose into eight-legged nymphs that require another blood meal in order to molt into adult ticks (Suss, 2003). Mating between adult ticks usually occurs on the last host when the male tick finds the engorged female. The adult male rarely feeds before mating with the engorging female (Kiszewski *et al*., 2001). After mating, the male dies immediately whereas the female dies a few weeks later, shortly after laying her eggs (Suss, 2003).

**Figure 2:** Life cycle of *Ixodes ricinus*. The eggs of *I. ricinus* hatch into six-legged larvae. The larvae remain mostly on the ground and usually attack small rodents and reptiles for blood meals. After blood feeding, the larvae molt into eight-legged nymphs. The nymphs also need a blood meal in order to molt into adult ticks. The adults usually seek a larger host where female ticks become engorged and mating with adult males takes place. The female ticks fall to the ground where they lay their eggs.
2.3 Environmental factors and tick ecology

Tick activity is determined by environmental factors like temperature and humidity, and climate change affects the distribution and abundance of ticks and TBPs (Jaenson & Lindgren, 2011). Ticks are sensitive to drought because they spend most of their lifetime close to the ground. The larvae are the most sensitive stage, and these usually stay close to the ground near the vegetation base, whereas adults can climb vegetation up to 1.5 meters high in their quest for a host (Mejlon & Jaenson, 1997). Nymphs begin questing at 7 °C, whereas larvae become active at 10 °C, and a humid spring (>10 °C) leads to simultaneous questing by larvae and nymphs resulting in enhanced chances of horizontal transmission of TBPs among co-feeding ticks (Randolph, 2000; 2011; Randolph et al., 1996; Zajkowska et al., 2006). In Europe, the dominant Ixodes species require at least 80% relative humidity in their microclimate in order to prevent fatal dehydration during the prolonged non-parasitic phase of their life cycle (Gray et al., 2009).

3. The pathogen

3.1 Flavivirus

TBEV is a flavivirus that belongs to the family Flaviviridae comprising four genera: Flavivirus, Pegivirus, Pestivirus, and Hepacivirus. The genus Flavivirus comprises 73 viruses that are classified into more than 50 species (Pierson & Diamond, 2013). The name flaviviridae (from flavus, “yellow” in Latin) originates from YFV, the first identified virus of the genus Flavivirus. YFV was first isolated in 1927 from the blood of a patient from Ghana (Pierson & Diamond, 2013). Dengue virus, West Nile virus, Zika virus, Japanese encephalitis virus, TBEV, and YFV are the principal flaviviruses that present serious threats to human health. Transmission of the flaviviruses mentioned above to vertebrates is primarily executed by mosquito or tick vectors. However, flaviviruses with no known arthropod vectors have also been described (Billoir et al., 2000; Gould et al., 2001). Although flaviviruses resemble each other in their genomic organization, antigenicity, and morphology, they exhibit diverse pathophysiological outcomes. Flavivirus-induced infections range from mild fever and headache to severe encephalitis, microcephaly, haemorrhagic fever, and shock syndrome (Gould & Solomon, 2008; Lindenbach et al., 2013; Pierson & Diamond, 2013).

3.2 Genomic organization of flaviviruses

Flaviviruses are enveloped icosahedral viruses of about 50 nm in diameter and contain a positive-sense single-stranded RNA (+ssRNA) genome of about 11 kb
with a 5′ cap, m7GpppAmN (Figure 3). The genome encodes a single polyprotein that is flanked by 5′ and 3′ non-coding regions (NCRs) of ~100 and 400–700 nucleotides (nt), respectively (Lindenbach & Rice, 2003). The polyprotein is processed by viral and host proteases into three structural proteins – capsid (C), precursor membrane (prM), and envelope (E) – and seven non-structural (NS) proteins – NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Heinz, 2003). The structural proteins provide the icosahedral shape, and the E protein is involved in receptor-mediated endocytosis, endosome fusion, and release of the virion. The polymerase and protease functions of flavivirus NS proteins are essential for virus replication and assembly (Lindenbach & Rice, 2003). In addition, flavivirus NS proteins are known to interfere with and evade host immune responses (Best et al., 2005; Laurent-Rolle et al., 2010; Lin et al., 2006; Liu et al., 2005; Munoz-Jordan et al., 2003; Rastogi et al., 2016; Werme et al., 2008). The 5′- and 3′NCRs contain complementary sequences that help genomic cyclization through long-distance base pairing to form a panhandle structure. The sequences and organization of flavivirus 5′- and 3′NCRs differ between the mosquito-borne, tick-borne, and no-known-vector viruses. However, several conserved regions, RNA secondary structures, and sequence repeats are common among flaviviruses, and the 5′- and 3′NCRs are important for replication, translation initiation, and packaging of the viral RNA (Chiu et al., 2005; Holden & Harris, 2004; Kofler et al., 2006; Markoff, 2003). The rigid RNA secondary structures and pseudoknots at the beginning of the 3′NCR protect the terminal ~0.3 to ~0.5 kb from exoribonuclease XRN1 that generates subgenomic flavivirus RNA (sfRNA) that is critical for the cytopathicity and pathogenicity of flaviviruses (Funk et al., 2010; Pijlman et al., 2008; Schnettler et al., 2014; Silva et al., 2010).

Figure 3: Schematic representation of a flavivirus particle and genome. A) Illustration indicating the arrangement of E protein dimers that form the herringbone-like icosahedral shape of a mature flavivirus particle (adapted from (Heinz & Stiasny, 2012)). B) Illustration showing the genomic organization of flavivirus.
3.3 Flavivirus replication cycle

The flavivirus life cycle involves attachment to the surface of the host cell, internalization of the virus, release of viral RNA, translation of viral proteins, viral RNA replication, packaging of virions, maturation, and release of progeny viruses. The replication cycle, as illustrated in Figure 4, can be divided into following main steps.

![Flavivirus replication cycle](image)

**Figure 4**: Flavivirus replication cycle. The virus is internalized through receptor-mediated endocytosis and forms an endosome. The fusion of viral and endosomal membranes results in virion release. The genome is translated into a single polyprotein that is further processed into 10 viral proteins that are important for replication complex assembly at the endoplasmic reticulum. RNA replication occurs at the replication complex, and immature virions are formed, which mature through the secretory pathway to generate mature virus particles.

3.3.1 Endocytosis

Flavivirus entry into the host cell requires interactions between the E glycolprotein and cell surface receptors. These interactions are mediated by multiple factors at the cell surface that facilitate receptor recognition and binding (Lindenbach et al., 2013). Previous studies have reported that glycoaminoglycans (GAGs), such as heparan sulfate, serve as attachment factors for several flaviviruses in different cell types (Chen et al., 2010; Chen et al., 1997; Germi et al., 2002; Kozlovskaia et al., 2010; Kroschewski et al., 2003; Lee & Lobigs, 2008; Mandl et al., 2001). The flavivirus E protein surface contains positively charged residues, whereas GAGs are negatively charged. The weak electrostatic interactions between the E protein and the sulfate group of GAGs concentrate the virus at the cell surface, facilitating recognition and binding to cellular receptors (Chen et al., 1997; Germi et al., 2002;
Kroschewski et al., 2003). Several other attachment factors exist because cells lacking GAGs can also be readily infected by flaviviruses (Kroschewski et al., 2003). Several putative entry receptors that are both cell-type and virus-specific have been described (Kaufmann & Rossmann, 2011; Perera-Lecoin et al., 2013; Smit et al., 2011), but this part of the flavivirus life cycle is still poorly understood. Flaviviruses enter host cells by clathrin-mediated endocytosis (Chu & Ng, 2004; Nawa et al., 2003; Ng & Lau, 1988; van der Schaar et al., 2008) in which clathrin-coated pits at the plasma membrane invaginate to form clathrin-coated vesicles inside the cell. The clathrin coat dissociates, and the virus-containing vesicle is transported within the cytoplasm and fused with other vesicles to form an early endosome that further matures into a late endosome (Smit et al., 2011; van der Schaar et al., 2008).

### 3.3.2 Membrane fusion

Fusion between the viral and endosomal membranes is pH dependent and is essential for release of viral RNA into the cytoplasm. Membrane fusion occurs primarily in late endosomes (van der Schaar et al., 2008), and the exposure of flaviviruses to the slightly acidic pH of the endosome induces conformational changes in E protein arrangements resulting in a shift from usual homodimers to fusion-active homotrimers. The E homotrimers facilitate fusion of viral and endosomal membranes by forming a fusion pore through which the nucleocapsid is released into the cytoplasm (Kaufmann & Rossmann, 2011; Smit et al., 2011).

### 3.3.3 Polyprotein processing

After uncoating of the nucleocapsid the flavivirus genome is released into the cytoplasm. The 5’ cap stabilizes the ssRNA genome and facilitates the initiation of translation. Translation of the single, long open reading frame (ORF) of the flavivirus generates a single polyprotein. The polyprotein is co- and posttranslationally processed into 10 viral proteins by viral and host proteases that cleave the protein junctions located at the cytoplasmic and ER lumen sides, respectively. The cleavage between the NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4A/2K, and NS4B/NS5 proteins is mediated by the viral NS2B/NS3 protease complex, whereas host signal peptidase cleaves the junctions between the C/prM, prM/E, E/NS1, and 2K/NS4B proteins (Lindenbach et al., 2013; Mandl, 2005; Mukhopadhyay et al., 2005).

### 3.3.4 RNA replication

Flavivirus RNA replication primarily occurs in small pouch-like invaginations called membrane vesicles (Ve) that are formed by virus-induced modifications of the ER membrane (Gillespie et al., 2010; Offerdahl et al., 2012; Welsch et al., 2009). The virus replication complex (RC), which comprises viral RNA, NS proteins, and host factors, is assembled at the Ve. Viral RNA, NS3, and NS5 localize on the cytoplasmic side of the Ve, NS1 localizes to the ER lumen, and
NS2A, NS2B, NS4A, and NS4B transverse the ER membrane (Apte-Sengupta et al., 2014; Welsch et al., 2009). A negative-strand RNA complementary to the genomic RNA is synthesized first, and this serves as a template to synthesize additional positive-strand RNA copies in a semi-conservative manner. Cyclization of the flavivirus genome is mediated by interactions between complementary sequences within the 5´ and 3´NCRs, and this is required for negative-strand RNA synthesis (Alvarez et al., 2005; Brinton, 2014; Khromykh et al., 2001). The asymmetric flavivirus replication produces about 10-fold more positive-strand RNA compared to negative-strand RNA (Cleaves et al., 1981; Muylaert et al., 1996). The newly formed positive-strand RNA performs three main functions: (1) it serves as mRNA to produce more virus proteins (translation), (2) it serves as a template for the synthesis of negative-strand RNA (replication), (3) it serves as the genome in the new virions (packaging). Besides positive-strand and negative-strand RNA, the generation of sfRNA has also been reported during flavivirus infections (Funk et al., 2010; Pijlman et al., 2008; Schnettler et al., 2014; Silva et al., 2010; Urosevic et al., 1997).

3.3.5 Virion production

Intracellular membranes are the main sites for the assembly and maturation of flavivirus virions. The newly synthesized genomic RNA is released through the Ve pore, and the flavivirus C protein localized on the cytoplasmic side of the ER near Ve pore or on lipid droplets captures nascent RNA to form the nucleocapsid that buds into the ER membrane to acquire the prM and E proteins (Apte-Sengupta et al., 2014; Lindenbach et al., 2013; Welsch et al., 2009). The premature virions are transported through the trans-Golgi network where prM and E undergoes acid-induced rearrangement and virus-containing vesicles are directed to the plasma membrane for exocytosis. The last step of virion maturation involves furin-like protease-mediated cleavage of prM protein that occurs during egress to generate mature infectious viruses (Brinton, 2014; Lindenbach et al., 2013; Pierson & Diamond, 2013; Welsch et al., 2009).

3.4 Tick-borne encephalitis virus

TBEV is known to cause severe central nervous system infection in humans. The virus is transmitted to humans through tick bites or by consuming unpasteurized milk or dairy products from TBEV infected animals. After TBEV infection, ticks carry the virus for the rest of their lives via transstadial transmission, and infected female ticks can transfer the virus to the next generation through transovarial transmission (Danielova et al., 2002; Karbowiak & Biernat, 2016). In addition, interstadial TBEV transmission from infected to susceptible ticks occurs while they co-feed on small rodents (Randolph, 2011; Randolph et al., 1999). These transmission routes make ticks both the vector and the reservoir for TBEV (Randolph, 2011). All three questing life cycle stages can attack
humans and transmit TBEV. Eu-TBEV is primarily transmitted by *I. ricinus*, whereas *I. persulcatus* is the main vector for Sib-TBEV and FE-TBEV transmission (Mansfield et al., 2009; Pierson & Diamond, 2013).

### 3.5 The NCRs of TBEV

The 5´ and 3´NCRs of flavivirus play important roles in viral replication, translation, virion assembly, and genomic stability during the flavivirus life cycle (Chiu et al., 2005; Gritsun et al., 1997; Kofler et al., 2006; Lindenbach et al., 2013). The RNA secondary structures, sequence motifs, and complementary sequences within the NCRs interact with viral and host factors to trigger flavivirus replication (Corver et al., 2003; Hahn et al., 1987; Khromykh et al., 2001; Kofler et al., 2006). The 3´NCR of TBEV can be divided into a core element (C 3´NCR) and a variable region (V 3´NCR) (Gritsun et al., 1997; Wallner et al., 1995). The terminal ~340 nt of the TBEV genome constitute the conserved C 3´NCR, whereas the V 3´NCR is heterogenic, both in its length and nucleotide sequence, among TBEV strains (Gritsun et al., 1997; Wallner et al., 1995). It has been proposed that during flavivirus replication, the V 3´NCR functions as an enhancer while the C 3´NCR encodes both enhancer and promoter regions (Gritsun & Gould, 2007). Fourteen stem loop (SL) structures have been predicted within the 3´NCR of TBEV strain Toro-2003. The terminal 1-5 SLs encode the promoter region, whereas SL6-14 encode the enhancer region (Elvang et al., 2011; Gritsun et al., 1997). Several Eu-TBEV strains contain a variable internal poly(A) tract within their V 3´NCRs, while a number of Eu-TBEV strains lack the poly(A) tract, and different truncations of the V 3´NCR exist (Asghar et al., 2014; Mandl et al., 1998; Wallner et al., 1995). Contrary to previous findings that the V 3´NCR of TBEV is dispensable for virus replication in cell culture and for virulence in mice (Mandl et al., 1998), it has been recently demonstrated that the V 3´NCR is an important virulence determinant of TBEV (Sakai et al., 2015; Sakai et al., 2014). The poly(A) tract within the V 3´NCR is proposed to contribute to TBEV virulence by inducing genomic instability that results in increased quasispecies diversity (Paper II). In addition, the sfRNA produced by incomplete exoribonuclease XRN1-mediated cleavage due to the conserved RNA secondary structures within the 3´NCR of flaviviruses is a critical virulence determinant in flavivirus infections (Funk et al., 2010; Pijlman et al., 2008; Schnettler et al., 2014; Silva et al., 2010).

### 3.6 Viral quasispecies

RNA viruses exist as swarms of unique variants called quasispecies where each variant contains a unique genome (Enquist & Racaniello, 2013). Spontaneous mutations occur in both RNA and DNA viruses, but the mutation rates of RNA viruses are 100 to 100,000 fold higher than those of DNA viruses (Condit, 2013).
The observed differences in mutation rates are associated with the proofreading ability of viral polymerases. The error-prone polymerases of RNA viruses that lack proofreading ability generate high genomic diversity in viral populations, and this facilitates virus evolution and adaptability in diverse environments (Condit, 2013; Sanjuan et al., 2010; Vignuzzi et al., 2006). TBEV quasispecies exist within ticks and mammals (Asghar et al., 2014; Luat le et al., 2014; Romanova et al., 2007; Ruzek et al., 2008), and the emergence of quasispecies from complementary DNA (cDNA) derived flaviviruses has also been reported (Mandl et al., 1998; Pletnev, 2001). The diversity of viral quasispecies facilitates virus survival in diverse hosts and contributes to its virulence. In vivo and in vitro passaging of TBEV suggests that host switching results in enhanced replication of the best-fit variants while the less-fit variants continue to replicate at a lower level and re-emerge after switching back to the original host (Romanova et al., 2007).
Methodological considerations

This section describes the basic principles behind the techniques used in this thesis, and discusses the rationale for selecting the specific methods. Detailed descriptions of the individual methods are available in the corresponding papers.

Tick sampling

To study tick population dynamics, accurate representative sampling of all of the questing forms is desired. Blanket dragging and flagging are the most commonly used methods for tick sampling. The former uses a wooden stick attached to one end of a white flannel blanket that is dragged over the vegetation with the help of a cord tied at both ends of the stick (Melik et al., 2007). Flagging involves waving a cotton flag over and through vegetation using a hand-held pole (Soleng & Kjelland, 2013). Contact between the tick and blanket is critical for sampling of questing ticks, and vegetation structure influences the selection of the best sampling technique. The mop-blanket technique developed here represents a slightly modified form of blanket dragging. The head of a dry mop was passed through the stitched end of a 75 cm × 80 cm woolen flannel blanket, and a tubular steel handle was attached to the mop head via a 360° swivel (Figure 5). The handle helps to drag the blanket at the desired height, and the swivel head allows the blanket to move in between the small spaces near the vegetation base.

Sampling was performed between 11:00 a.m. and 6:00 p.m., but was avoided during rainy days. At each sampling site we randomly selected 10 plots (3 m × 3 m each) and swept the mop-blanket in right to left and up and down motions at normal walking speed (approximately 1 m/s). The area of each sampling plot was limited to 9 m² because the risk of brushing off and dislodging ticks from the blanket increases with dragging distance. Ticks were harvested from both sides of the blanket after each sweep. Ticks from each sampling site were frozen in liquid nitrogen and stored at −80°C for later analysis. Our tick sampling experience suggested that the mop-blanket technique is substantially better than blanket dragging and flagging for representative sampling of all questing life cycle stages of *I. ricinus*.
RNA extraction and cDNA synthesis

RNA is a very unstable molecule that is vulnerable to enzymatic digestion by ribonucleases and to temperature dependent degradation, which is at a maximum at $\geq 37 \, ^\circ\text{C}$ and a minimum at $\leq -80 \, ^\circ\text{C}$. Thus RNA is usually converted to more stable cDNA for detection and identification of different TBPs within ticks or other biological specimens. For RNA isolation, a ribonuclease-retarding buffer is used to homogenize the pathogen-containing sample, and after sequential purifications the RNA is usually precipitated with ethanol. The extracted RNA can be used for cDNA synthesis, primer extension, Northern blotting, etc. For cDNA synthesis, a specific primer complementary to the 3’ end of the virus or random hexamer primers are used. RNA is heated briefly to eliminate RNA secondary structures, and reverse transcriptase ensures efficient cDNA synthesis. The resulting cDNA can be used as a template for polymerase chain reaction (PCR).

Quantitative reverse transcription PCR

Reverse transcription, or cDNA synthesis, refers to DNA production from an RNA template. The combination of reverse transcription and PCR in real time (qRT-PCR) can detect and quantify the number of specific RNA copies in a given sample after each round of amplification. qRT-PCR is based on the detection of fluorescence signal from a reporter molecule. Two established tech-
niques, SYBR Green assay and TaqMan assay, require reporter molecule binding to the double-stranded DNA or to a specific template between the two primers, respectively, to produce a fluorescence signal. Fast speed, high sensitivity, and specificity make qRT-PCR a method of choice for quantitative detection of TBEV in a given sample. In addition, sequencing the PCR product provides additional confirmation and facilitates TBEV strain characterization by phylogenetic analysis.

Nested PCR

Nonspecific primer binding is a principal limitation of classical PCR, and such binding might result in amplification of more than one DNA product. Nested PCR is a modified form of the standard PCR technique that allows for highly specific amplification of the target sequence. It utilizes two specific primer sets in two successive PCR reactions to amplify a single DNA fragment. The first primer set, the outer forward and outer reverse primers, is used to amplify a sequence of interest from a specific DNA template during PCR-1. The product from PCR-1 is used as the template in PCR-2 to amplify the fragment of interest using the second/nested primer set, i.e. the inner forward and inner reverse primers. The nested primers usually bind inside the product from PCR-1 to generate a shorter and more specific PCR-2 product. Nested PCR is used to detect extremely low TBEV quantities within a given sample by specific amplification of conserved sequences within the TBEV genome. The two successive PCR amplifications contribute to the higher sensitivity (mainly PCR-1) and specificity (mainly PCR-2) of the nested PCR. Sequencing the purified PCR-2 product helps to characterize the TBEV strain by phylogenetic analysis.

Phylogenetic analysis

Phylogenetic analysis is an approach to estimate the evolutionary history and relationships among a group of genes or organisms, and it infers relations and times of divergence between organisms that originated from a common ancestor. Evolutionary models that use inheritable traits like nucleic acid sequence or morphology are usually employed to perform the analysis. The evolutionary history is usually presented in the form of a tree-like diagram called a phylogenetic tree or dendrogram where organisms or genes with shared traits cluster together to form distinct clades. The nodes in a dendrogram suggest distinct evolutionary bifurcations, and branch lengths represent evolutionary distance and tips indicate the earliest or latest evolutionary lineage. Phylogenetic analysis is a vital technique in evolutionary biology, biodiversity, and genomics.
**In vitro transcription**

In vitro transcription refers to RNA synthesis from a linearized DNA template under laboratory conditions. The technique does not require cells or organisms, thus making it especially suitable for working with RNA that would be lethal to cells or organisms. The system comprises a specific pair of a bacteriophage promoter and its RNA polymerase, which results in exclusive synthesis of the RNA of interest. T3, T7, and SP6 are the commonly used promoters for in vitro transcription. In vitro transcription can synthesize micrograms to milligrams of RNA transcripts up to several kilobases in size. In vitro-transcribed RNA with desired sequence modifications can be employed in different experimental settings such as in vitro translation, functional ribozymes, infections with RNA transcripts, rescue of cDNA-based infectious viruses, microinjections, RNA probe hybridization, etc.

**Reverse genetics**

In contrast to forward genetics that investigates genetic fingerprints of observed phenotypes, reverse genetics goes in the direction from genotype to phenotype. Owing to advances in sequencing technology, whole genome sequences of a large number of organisms are available today. Using a reverse genetics approach one can investigate individual gene functions and the effect of a single nucleotide mutation in a gene of interest and can generate new candidates for vaccines, gene therapy, and oncolytic vectors (Condit, 2013). In general, any virus can be rescued with reverse genetics techniques. However, the rescue principles are dependent on the genomic structure, replication site, and replication strategy of the given virus. The genomes of all +ssRNA viruses are infectious, and to rescue specific mutants of TBEV, which is a +ssRNA virus, the desired mutations can be engineered into the TBEV cDNA clone. The transfected cDNA clone or in vitro-transcribed RNA is eventually translated to express viral proteins that are essential for initiating virus replication in transfected cells followed by recovery of viruses identical to the transfected cDNA or RNA sequence.

**Construction and rescue of cDNA-based infectious clones of TBEV**

Our group has previously sequenced and extensively characterized the complete genomic sequence of Eu-TBEV strain Toro-2003 from a TBEV natural focus in Torö (58°49´ N, 17°50´ E) in southern Stockholm, Sweden (Elvang et al., 2011; Melik et al., 2007), and we have established a technique to rescue infectious clones of Toro-2003 (Paper II). In addition, infectious clones with a reporter and
chimeric virus were also generated that will be used in future studies. The basic strategy for constructing the infectious clones is illustrated in Figures 6–8 in a stepwise manner where nucleotide positions correspond to Toro-2003 (GenBank Accession no. DQ401140.2). In the illustrations, thick blue lines correspond to TBEV sequences, fragments of interest are indicated by thin green lines, thin double-lines represent the vector backbone, and unique restriction enzymes are indicated as perpendicular lines. All of the PCR-amplified fragments were cloned into pcDNA™3.1/V5-His-TOPO® (Invitrogen) using standard molecular biology procedures such as ligation, partial digestion, transformation, and restriction analysis. The products of PCR amplification and DNA ligation were confirmed by restriction enzyme analysis and/or sequencing (Eurofins MWG Operon, Ebersberg, Germany).

Step I: Ligation of the genomic sequence encoding the structural proteins and NS1 (pspOMI)

In this step, the TBEV genomic fragment 121/2460 was extended to 121/2584. The CprME in Topo® plasmid was digested with HindIII followed by gel purification of the resulting 2272 bp fragment. The E-NS3 in Topo® plasmid was digested with HindIII/pspOMI to obtain a 238 bp fragment and the 5421 bp vector backbone. The above three fragments were ligated to obtain CME+NS1( pspOMI) in Topo® plasmid that contained the genomic fragment encoding C, prM, E, and a small part of NS1 (Figure 6).
Figure 6: Strategy used for molecular cloning of the genomic fragment containing the structural genes and partial NS1 gene.

Step II: Ligation of the 5’NCR sequence with the sequence that encodes the structural proteins

The Toro-2003 luciferase sub-genomic replicon (Melik et al., 2012) was used as the template to amplify the 1/213 fragment using a 5’NCR forward primer (5´-AGA TTT TCT TGC ACG TGC-3´) and a reverse primer that introduced a MluI site (5´-ACG CGT CTT CGT TGC GGT CTC TTT CGA CAC TCG TCG AGG GGG AC-3´). The PCR protocol included 25 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 3 min. The amplified fragment was cloned into pcDNA™3.1/V5-His-TOPO® (Invitrogen) to generate 5´NCR-C in Topo®. The NdeI-MluI fragment from 5´NCR-C in Topo® was ligated with the MluI fragment from CME+NS1(bspOMI) in Topo® (step I) and backbone of the vector (MluI-NdeI) to generate the 5´NCR-NS1(bspOMI) in Topo® (Figure 7).
Step III: Construction of the full-length TBEV cDNA

The 5´NCR–NS1(pspOMI) in Topo® plasmid (step II) was digested with KpnI/pspOMI to obtain a 2620 bp fragment (containing TBEV 5´NCR–NS1), and the TBEV replicon was digested by pspOMI/PvuI to obtain an 11,924 bp fragment (containing TBEV NS1–3´NCR). The two fragments were ligated to generate a single fragment that served as the template for the subsequent PCR (Figure 8). The complete TBEV genome was amplified using a forward primer containing the SP6 promoter sequence followed by the 5´NCR sequence (5´-ATT TAG GTG ACA CTA TAG ATT TTC TTG C-3´) and a reverse primer (5´-AGC GGG TGT TTT TCC GAG TCA CAC-3´) complementary to the end of the 3´NCR of TBEV.
**Figure 8:** Molecular cloning strategy to generate the full-length cDNA clone of Toro-2003.

**Step IV: Rescue of the TBEV infectious clone**

Capped TBEV RNA transcripts were generated by *in vitro* transcription. The RNA was transfected into a mixed (1:1) HEK293 and Vero B4 cell population where HEK293 cells provided transfection advantage and Vero B4 cells provided replication advantage. Cells were monitored for cytopathic effects, and the infectious clone was harvested after appearance of such effects.

**Confocal laser scanning microscopy**

Confocal laser scanning microscopy (CLSM) is a standard technique for capturing high-resolution optical images at variable depths of a living or fixed biological sample. The technique utilizes different conjugated fluorophores such as green fluorescent protein (GFP), which can be excited at specific wavelengths to generate fluorescence. A laser scans the specimen at a desired depth to generate a confocal plane, and images with better contrast are captured. This optical sectioning enables 3-D simulation of small organelles within a single cell. High-definition images are obtained by reducing background fluorescence and thus increasing the signal to noise ratio. CLSM of living specimens can reveal spatiotemporal dynamics of target proteins in their functional states. However, the risk of overlapping or crossover emission is high when two or more fluorophores with overlapping emission spectra are used. This problem can be resolved by using fluorophores with distinct excitation-emission spectra.
METHODOLOGICAL CONSIDERATIONS

Plaque assay

Plaque assays are a standard method to determine virus concentration. Compared to qRT-PCR that quantifies the number of viral RNA copies in a sample, the plaque assay only quantifies infectious viruses thus excluding defective viral particles. The assay involves infection of a confluent monolayer of cells with 10-fold virus dilutions. The virus inoculum is removed after 1 h, and cells are covered with a semisolid medium to restrict virus dissemination. During incubation, virus-infected cells lyse, and the released viruses spread to neighbouring cells to form plaques. After the virus-dependent incubation period, the cells are fixed, permeabilized, stained with virus-specific antibodies, and visualized by a special dye. The number of plaque-forming units (pfu) is counted, and this corresponds to the number of infectious virus particles in the original sample. The virus concentration is calculated as pfu/mL considering the dilution factor. In paper II, we performed a plaque assays to determine the TBEV concentration required to calculate the specific multiplicity of infection for infecting cells or mice.

Next generation sequencing

Next generation sequencing (NGS) or deep sequencing refers to high-throughput, massively parallel, exceptionally high-speed sequencing of DNA or RNA. In comparison to conventional Sanger sequencing, NGS is inexpensive, requires little input DNA, and is capable of paired-end sequencing of single DNA fragments (Mardis, 2008). However, Sanger sequencing was the method of choice for sequencing the poly(A) tract of Saringe-2009 and Torö-38A in papers I and II, respectively. This was due to the limitation of NGS in sequencing long stretches of homopolymeric nucleotide sequences like poly(A) tracts (Quail et al., 2012). NGS can identify mutational spectra around a single nucleotide or across the complete genome of an organism, and we performed NGS in papers II and III to identify the mutational structure of TBEV quasispecies present within mammals and ticks, respectively. It would have been impossible for us to conduct these studies without NGS considering the time and cost to do so.

We used the Nextera® XT DNA library preparation kit (Illumina®) because it is the best kit for sequencing small genomes like TBEV, and all work was performed on a Miseq NGS platform (Illumina®). The system generates paired-end reads. The method utilizes a transposome-mediated tagmentation reaction that cleaves the input DNA into small fragments and simultaneously adds tags to the ends of each fragment. In the next step, specific adaptors (primer binding sequences, indexes, and regions complementary to flow-cell oligonucleotides) are added to the tagged fragments by reduced cycle amplification. The flow cell used for sequencing contains two types of oligonucleotides embedded in its
polymer-coated glass surface. Hybridization occurs between the flow-cell oligonucleotide and the complementary adaptor sequence in the DNA fragment, and this is followed by synthesis of the complementary strand. The template DNA fragment is removed after denaturation, and the free end of the newly synthesized complementary strand hybridizes/bridges with the second oligonucleotide embedded in the flow cell. Each strand is clonally amplified by bridge amplification of the DNA fragment to generate a cluster. This process is called cluster generation, and millions of clusters are generated simultaneously. After this step, reverse strands are cleaved and washed away. Sequencing begins with extension of the first sequencing primer where addition of each nucleotide emits a specific fluorescence signal. For the second strand sequencing, a complementary strand of the forward strand is generated by bridge duplication, and the forward strand is cleaved away followed by reverse strand sequencing. The read products are washed away after sequencing. Sequencing was performed overnight, and about 1 million reads were generated.

**Single nucleotide polymorphisms**

Single nucleotide polymorphisms (SNPs) are single nucleotide mutations at specific points in the genomic sequence of an organism that persist in >1% of a given population. SNPs provide a fitness advantage for TBEV replication in diverse cellular environments. We used NGS to analyse the occurrence of SNPs within TBEV quasispecies populations from questing ticks, cell cultures, and mammals (papers II and III). For SNP analysis, NGS reads with average quality scores >30 were used and PCR duplicates were removed. The residual filtered reads were aligned against respective reference genomes, and the percentages of SNPs for each nucleotide were calculated.

**Mutation frequencies**

Mutation frequency analysis reflects the genomic variation of an organism in a given sample, and we calculated mutational frequencies (papers II and III) to reveal the TBEV quasispecies diversity within questing ticks and mammals. RNA viruses have high mutation rates that are usually presented as substitutions per nucleotide per generation. Because the correct estimation of mutation rate requires the exact number of replication cycles, we were satisfied with presenting the mutation frequency as simply substitutions per nucleotide as long as we acknowledged the exact passage history of each virus. For all mutation frequency calculations, the sum of mutations within a given gene was divided by the sum of nucleotides sequenced for respective gene.
RNA folding

The ssRNA of TBEV is known to fold by base pairing between complementary regions in the genome. SLs are the most common RNA secondary structures, and they are known to interact with different cellular proteins. Complementary base pairing between nucleotides within a short stretch of RNA results in a double-stranded stem structure, and the unpaired nucleotides located between the complementary regions form single-stranded loops. There exists a total of 4 SLs in the 5´NCR and 14 SLs in the 3´NCR of TBEV strain Toro-2003 (Elvang *et al.*, 2011). The complementary cyclization sequences located at the termini are essential to form RNA secondary structures that facilitate translation initiation, and replication of TBEV. We used the RNAfold web server (Zuker, 2003) – which utilizes RNA-folding algorithms to predict multiple foldings for a given RNA sequence using thermodynamic methods – for TBEV genome folding analysis in paper II.

Cell culture

Cell culturing refers to the cultivation of eukaryotic cells under controlled conditions. Cells are usually grown in a humidified environment at the appropriate temperature in sterile flasks/plates supplemented with a special growth medium containing small amounts of antimicrobial agents to protect the cells from microorganisms. Cell culture is an important technique that mimics the *in vivo* environment, hence allowing basic experiments to be performed without using whole organisms. Primary cell cultures refers to the cells that have been grown directly from a tissue, while immortalized cell cultures are those in which cells can grow and divide infinitely as long as optimal growth conditions are maintained. Primary cell cultures better reflect the *in vivo* state, whereas immortalized cultures generate a more homogenous source without the expense of live animals. In paper II, we used the immortalized cells Vero B4 that are interferon deficient which makes them ideal for TBEV infection, HEK293 cells that offer higher transfection efficiency, and A549 cells that exhibit normal immune responses to viral infection and are useful for studying the replication of TBEV when challenged by the immune system.

Ethical considerations

The TBEV strains Saringe-2009 and Habo-2011 (paper I) were identified in blood-feeding ticks that were deposited with the STING study by tick-bitten humans who provided written consent before entering the study. The regional ethics committee in Linköping granted the ethical approval (M132-06) for the STING study following the principles expressed in the Declaration of Helsinki.
For animal experiments (paper II), the mice were housed and handled in accordance with good animal practice as defined by the Federation of European Laboratory Animal Science Associations. All animal experiments were performed in compliance with the German animal welfare law (TierSchG BGBl. S. 1105; 25.05.1998) and were approved by the responsible state office under permit number AZ 33.9-42502-04-11/0528.
Results and discussion

Paper I

Over the past several decades, an increase in the prevalence and incidence of TBE has been reported in Scandinavia (Jaenson et al., 2012b). The rapidly escalating situation demands thorough epidemiological and clinical investigations. TBE has been a notifiable disease in Norway since 1975, but no complete genome sequence of Norwegian TBEV was available until we published such a sequence in 2014. In this study we sequenced and phylogenetically characterized two TBEV genomes – Mandal-2009 and Saringe-2009 – where Mandal-2009 represents the first reported TBEV genome from Norway.

Most of the available TBEV genome sequences in the database have been generated after virus isolation by conventional enrichment procedures such as cultivation in suckling mouse brain or in cell culture. However, it has been clearly demonstrated that such cultivations can alter the genetic composition of the virus (Mandl et al., 1998; Romanova et al., 2007; Whetstone et al., 1989). The two TBEV genomes characterized in this study were generated directly from I. ricinus, thus providing TBEV genomes as they were found in nature. Mandal-2009 was sequenced from a pool of 10 questing nymphs, and Saringe-2009 was generated from a single nymph that had been sucking blood for >60 h on a human male. Phylogenetic analysis predicted a strong evolutionary relationship between Mandal-2009 and Saringe-2009.

The V 3’NCR of TBEV is highly heterogenic among Eu-TBEV strains, and the Neudoerfl and Hypr strains represent the longest and shortest V 3’NCRs, respectively (Wallner et al., 1995). Our genomic analysis showed that Mandal-2009 contains a truncated V 3’NCR (Figure 9) similar to the highly virulent Hypr strain, and this might be an important virulence determinant of the Norwegian strain. To our knowledge, Mandal-2009 is the first truncated Eu-TBEV variant identified in nature, and this strain might have arisen due to TBEV genomic instability within the tick vector or it might have been transmitted from Eastern Europe. Because our phylogenetic analysis revealed a closer relation between Mandal-2009 and the Swedish Saringe-2009 strain than between Mandal-2009 and Hypr, we hypothesized that Mandal-2009 evolved by truncation of longer Scandinavian TBEV variants.

In-depth analysis of Saringe-2009 showed a different genomic organization compared to Mandal-2009, and this was especially evident in the different
lengths of the V 3´NCR (Figure 9). Although TBEV quasispecies have been shown to exist within ticks and mammals (Romanova et al., 2007; Ruzek et al., 2008), the existence of different quasispecies in the different stages of the natural life cycles of RNA viruses was challenged (Holmes, 2009). Saringe-2009 contained a highly variable poly(A) tract in the V 3´NCR. The variability in the poly(A) tract of Saringe-2009 revealed the existence of TBEV quasispecies in nature, suggesting a putative shift in the quasispecies pool when the virus switches between invertebrate and vertebrate environments. We sequenced and analysed the V 3´NCR of six additional TBEV strains acquired from human blood and from questing and blood-feeding ticks, and four of these strains showed little variation within their V 3´NCRs. We hypothesized that the observed variability could be a key determinant of TBEV virulence, and additional studies of the V 3´NCR and the poly(A) tract in different TBEV strains and infectious clone models should be performed.

Figure 9: Graphical summary of paper I showing schematic view of the 3´NCR of Mandal-2009 and Saringe-2009.

Paper II

In Paper I, we observed variability in the poly(A) tract length of TBEV strain Saringe-2009. In paper II we extended our investigations to study the effect of poly(A) length variability on replication and virulence of Eu-TBEV. We recovered two cDNA-based infectious clones of Eu-TBEV strain Toro-2003 – Torö-6A and Torö-38A, with short and long poly(A) tracts, respectively. The long poly(A) tract of Torö-38A attenuated virus replication in cell culture as
RESULTS AND DISCUSSION

indicated by small, diffuse plaques and poor replication in Vero B4 cells. However, Torö-38A showed significantly higher neuroinvasiveness and neurovirulence compared to Torö-6A in C57BL/6 mice (Figure 10), which indicated a putative pathophysiological role of the longer poly(A) tracts detected in Eu-TBEV variants. A similar role for a long poly(A) tract in virulence has also been demonstrated for FE-TBEV (Sakai et al., 2015).

TBEV is a neurotropic virus. We had shown the existence of TBEV quasispecies within a single blood-feeding tick (Paper I), and researchers have suggested that variants with neurotropic properties within the quasispecies population of transmitted TBEV might become dominant in mammalian hosts and cause neurological symptoms (Ruzek et al., 2008). We investigated the role of the poly(A) tract in quasispecies development in mice using conventional sequencing and NGS. Sequencing of the input viruses and those isolated from mouse brain showed high variability within the V 3’NCR of the Torö-38A virus with the long poly(A) tract compared to Torö-6A. A relation between quasispecies diversity and the neurotropism and neuropathogenicity of poliovirus and YFV has been described (Beck et al., 2014; Vignuzzi et al., 2006), and the overt quasispecies diversity of Torö-38A compared to Torö-6A might be behind the observed differences in neuroinvasiveness and neurovirulence. Contrary to the general assumption that cDNA-derived infectious clones generate viruses identical to the parental clones (Condit, 2013), both the NGS and Sanger sequencing analysis revealed that diverse viral variants developed rapidly within the TBEV pool and were present already at passage 0 of recovered viruses.

We performed SNP analysis to investigate the evolution of TBEV quasispecies during both cell culturing and replication in mice. The results of this analysis suggested that TBEV quasispecies evolve by selection of existing variants as well as by the emergence of new mutations. We identified host-specific genetic divergences in TBEV quasispecies that might reflect the selection pressure exerted by the different hosts. Cell culturing for five passages resulted in mutations in the E protein of Torö-6A, and these mutations were predicted to increase the net positive charge on the virus surface. Such mutations enhance virus entry into certain cell types through improved binding to the heparan sulfate receptors (Mandl, 2005). In addition, we investigated the genomic stability of both viruses by calculating the mutation frequencies for individual genes. The average mutation frequencies across the entire ORF were similar for both of the viruses despite significant differences in replication rate, indicating that the RNA replication rate is independent of RNA polymerase fidelity. We observed noticeable differences between the mutation frequencies of individual genes, and this observation highlighted the importance of using the complete ORF instead of a specific gene for comparing mutation frequencies among different viruses.

SLs are the most common RNA secondary structures, and they are involved in several important interactions. The 3’ NCR of the TBEV strain Toro-2003 has
been shown to form 14 SLs where SL1–5 constitute the promoter region and SL6–14 were identified within the enhancer region of the 3´NCR (Elvang et al., 2011; Gritsun et al., 1997). RNA folding of the Torö-6A and Torö-38A genomes and their variants with heterogenic V 3´NCRs revealed that SL14 was the only RNA secondary structure affected by the poly(A) insertions or deletions observed within the V 3´NCR. We hypothesized that the intact SL14 might be critical for virus recognition by host immune system components. Our findings demonstrated that the length of the poly(A) tract is critical in determining the quasispecies diversity and virulence of TBEV, and to our knowledge this was the first study that demonstrated the poly(A) tract to be a virulence determinant for Eu-TBEV.

![Graphical summary of paper II showing schematic view of Torö-6A and Torö-38A rescue by reverse genetics and virulence in mice.](image)

**Figure 10**: Graphical summary of paper II showing schematic view of Torö-6A and Torö-38A rescue by reverse genetics and virulence in mice.

**Paper III**

Our previous work (**Paper I and II**) had demonstrated TBEV quasispecies diversity within a single blood-feeding tick and in TBEV strains isolated from human blood, cell cultures, and mice. In **Paper III** we investigated the population structure of TBEV quasispecies within questing *I. ricinus*. The geographical distribution of TBEV displays a patchy configuration where each patch represents a TBEV hotspot (Dobler et al., 2011; Suss, 2011). TBEV is endemic in central and southern Sweden, and TBEV hotspots have been shown to be concentrated in the Stockholm archipelago (Pettersson et al., 2014). Despite the availability of a reasonable number of TBEV sequences, our knowledge regarding TBEV sequences in nature is still quite limited. This is because most of the available TBEV genomes have been sequenced after cultivation in cell culture or in mice. Our group had identified a new TBEV hotspot at Torö (58°49´ N, 17°50´ E) in southern Stockholm in 2003 and characterized the first TBEV genome, Toro-2003, from this hotspot (Melik et al., 2007). In this study, we
sequenced and characterized two additional TBEV strains from Torö, JP-296 and JP-554.

JP-296 and JP-554 were sequenced directly from the total RNA of questing male *I. ricinus* without conventional enrichment procedures. Phylogenetic analysis predicted a strong evolutionary relationship between JP-296 and JP-554. Interestingly, both of the new strains grouped with one Norwegian strain, Mandal-2009 (*Paper I*), and two Swedish strains, Toro-2003 (the prototypic strain from Torö) and Saringe-2009 (*Paper I*). All five strains appeared to have originated from the same ancestor, and this strengthened the previously described Scandinavian clade (*Paper I*). In line with our previous findings in *Paper I*, these observations also supported a strong phylogeographic relation among the Scandinavian strains sequenced directly from the tick vector.

Eu-TBEV strains have highly conserved genomes with the exception of the highly variable V 3′NCR. We aligned the V 3′NCR of the five Scandinavian strains to identify variations among the strains. Mandal-2009 showed a highly truncated V 3′NCR, whereas JP-296 and JP-554 presented with a V 3′NCR organization identical to Toro-2003, which indicated the stability of Eu-TBEV within a natural TBEV focus. We investigated the TBEV quasispecies structures of JP-296, JP-554, and Mandal-2009 by NGS, and the strains with an intact V 3′NCR (JP-296 and JP-554) had significantly higher quasispecies diversity compared to Mandal-2009, which has a truncated V 3′NCR (Figure 11). The viral RNA-dependent RNA polymerase incorporates one mutation per genome per replication cycle (Sanjuan *et al.*, 2010), and we proposed that the higher quasispecies diversity of JP-296 and JP-554 could be due to higher virus loads (Ct = 16.52 and 17.21, respectively) compared to Mandal-2009 (Ct = 24). The low virus loads in the case of Mandal-2009, could be due to: 1) dilution effect because of pooling (n = 10 nymphs), 2) slower replication rate, or 3) comparatively less time spent in the tick after infection, assuming that all the three tick samples were infected at the same stage of tick life cycle, because Mandal-2009 was isolated from nymphs while JP-296 and JP-554 were isolated from adult males. In addition, NGS showed that 40% of the SNPs were common between quasispecies populations of the two new Torö strains, and thus indicating TBEV persistence within a TBEV hotspot. To our knowledge this was the first study that compared quasispecies dynamics of TBEV strains with intact and truncated V 3′NCRs and demonstrated similarities in TBEV quasispecies populations within a natural focus.
After investigating TBEV quasispecies configurations in the laboratory and in nature (Papers I–III), we focused on the study of tick population dynamics. *I. ricinus* is a common tick in Europe and represents the main vector for Eu-TBEV transmission to humans and livestock. In this study we established a standard technique for the accurate representative sampling of all three questing stages of *I. ricinus* to estimate tick abundance in southern Stockholm, Sweden. Our mop-blanket technique is unique, and the mop handle helps to drag the blanket at desired heights thus facilitating adjustment to local conditions. The swivel head provides 360° rotation and facilitates sampling from narrow spaces near the vegetation base to assure representative sampling of the three questing stages. We sampled ticks from randomly selected 3 m × 3 m plots to minimize the risks of tick dislodgment and brushing off (Estrada-Pena & de la Fuente, 2014). We found variations in tick abundance among sampling sites, which varied from 0.1 to 0.9 ticks per m².

TBEV is maintained in nature by enzootic cycling between tick vectors and vertebrate hosts (Labuda & Randolph, 1999; Moshkin *et al.*, 2009). Co-feeding among ticks is an important route for the horizontal transmission of several TBPs (Gern & Rais, 1996; Labuda *et al.*, 1993; Zemtsova *et al.*, 2010), and the co-occurrence of infected and non-infected ticks is a requirement for TBEV transmission by co-feeding. We analysed the effect of environmental and local landscape factors on population dynamics and the probability of co-occurrence of larvae, nymphs, and female *I. ricinus* (Figure 12). The probability of co-
occurrence was twice as high at the beginning of summer compared to later in the season and decreased significantly with vegetation height ($p < 0.01$). Larvae and nymphs showed peak questing activity in June, whereas the questing peak for adult ticks was observed in July, and temperature exerted a significant negative effect on adult ticks ($p < 0.001$). A significant positive relationship was observed between humidity and the abundance of questing nymphs ($p < 0.05$).

Landscape variables influence population dynamics of arthropod vectors and their vertebrate hosts that in turn affect the distribution and transmission of vector-borne pathogens (Lambin et al., 2010; Medlock et al., 2013). We observed a negative effect of vegetation height on the abundance of questing ticks. The amount of coniferous forest, deciduous forest, and open water around the sampling sites showed a significant negative effect on *I. ricinus* abundance and the co-occurrence of larvae, nymphs, and adults. However, these landscape effects were observed at very local scales and diminished quickly within 1 km around the sampling sites. This suggests that extra caution should be taken when extrapolating local findings from similar studies to larger spatial scales. Additional studies including extended, randomized tick sampling and host biodiversity are required for better understanding the effects of landscape factors on ticks and TBEV dynamics.

**Figure 12:** Graphical summary of paper IV showing effect of environmental and local landscape factors on the probability of co-occurrence of larvae, nymphs, and female *Ixodes ricinus*. 
Concluding remarks and future perspectives

TBEV affects between 10,000 and 15,000 people in Europe and Asia each year (Dobler, 2010). The increasing distribution range of *I. ricinus* and TBEV in Scandinavia, reaching 200–300 TBE cases in Sweden annually, demands extensive studies to investigate the hidden determinants of increased tick abundance, TBEV virulence, and TBEV persistence in nature. The continuous increase in the number of TBE cases is in accordance with constantly emerging natural foci in Scandinavia (Brinkley *et al.*, 2008; Pettersson *et al.*, 2014), and the synchronized seasonal activity of *I. ricinus* larvae and nymphs has been proposed to be an important condition for the existence of natural foci (Labuda & Randolph, 1999; Randolph *et al.*, 1999).

We developed a unique method for the representative sampling of all life cycle stages of ticks, and we studied the effect of environmental factors on the co-occurrence of larvae, nymphs, and female *I. ricinus* (Paper IV). One of our main findings was that temporal synchrony in questing life cycle stages of the tick occurred in the middle of the summer. This might lead to a higher risk of TBEV infections at the end of summer and in the fall when considering co-feeding as the main horizontal transmission route of TBEV among ticks. Another important finding in Paper IV was that the effect of environmental factors, including forest cover and open water, was extremely local. This suggests that the site-specific environmental conditions within local natural TBEV foci are very important for hosts, ticks, and TBPs. Our study could not accurately identify risk factors, and we need additional studies with standardized tick sampling from natural endemic foci to better understand the relationship between local and regional environmental conditions and the epidemiology of TBPs.

Despite the availability of a significant number of TBEV genomes, our understanding of TBEV persistence and evolution in nature is still lacking. This is due to a dearth of TBEV genomic sequences that exist in nature. Most of the TBEV genomes available today have been sequenced after cultivation in cell cultures and/or mouse brain. Such cultivations can alter the genetic structure of TBEV as indicated by infectious clones of Toro-2003 that developed specific mutations after a single passage in cell culture or animal models (Paper II). Similar changes in the genetic composition of bovine herpes virus 1, West Nile virus, and TBEV after a single passage in their respective hosts have also been reported (Dridi *et al.*, 2015; Romanova *et al.*, 2007; Whetstone *et al.*, 1989). We contributed to the understanding of TBEV evolution in nature by sequencing four TBEV genomes – Mandal-2009, Saringe-2009, JP-296, and JP-554 – directly
from the tick vector (Paper I and III). However, additional studies exploring novel TBEV genomes from tick vectors and vertebrate hosts sampled at natural foci are critical for a better understanding of TBEV persistence and evolution in nature. In addition, sequencing TBEV genomes directly from a tick vector or vertebrate host in nature and the isolates of the same viruses after laboratory cultivation will help to differentiate the genetic changes that emerge under natural conditions from laboratory-induced mutations.

TBEV causes severe encephalitis with long-lasting sequelae or permanent brain damage. However, little is known about the underlying mechanism of TBEV virulence. When the work for this thesis began, the V3′NCR and poly(A) tract of TBEV were considered dispensable for TBEV replication and virulence in mice (Hoenninger et al., 2008; Mandl et al., 1998; Ruzek et al., 2008), but the V3′NCR was predicted to play an important role during TBEV replication in nature (Hayasaka et al., 2001; Mandl et al., 1998; Melik et al., 2007). Contrary to the former view, it has recently been demonstrated that the V3′NCR and poly(A) tract modulate the virulence of FE-TBEV (Sakai et al., 2015; Sakai et al., 2014). Our work in Paper II established that the length of the poly(A) tract is an important virulence determinant of Eu-TBEV, and the virus strain with the longer poly(A) tract exhibited enhanced neuroinvasiveness and neurovirulence in a mouse model. RNA folding predictions of TBEV genomes showed that the long poly(A) tract abolished a SL structure at the beginning of the 3′NCR that could be important for virus-host interactions. However, additional studies using TBEV infectious clones carrying specific SL deletions or different lengths of poly(A) tract are required to further characterize the explicit RNA secondary structure responsible for the observed virulence and to identify interacting partners within host cells. In addition, we are currently generating chimeric viruses using our established technique to study the role of specific genomic regions in TBEV virulence. We are also working to rescue infectious clones of other flaviviruses to study and compare the host immune response to different flavivirus infections.

Before my PhD work, TBEV was known to exist as populations of mixed variants (quasispecies) both in ticks and mammals (Romanova et al., 2007; Ruzek et al., 2008). However, the existence of functional quasispecies within the natural life cycles of RNA viruses had been challenged (Holmes, 2009). In this thesis we have shown, for the first time, the existence of TBEV quasispecies within questing and blood-feeding I. ricinus (Papers I and III). In addition, we have demonstrated changes in TBEV quasispecies structure after cultivation in cell culture and in mice (Paper II). Mutation frequency analysis (Beaucourt et al., 2011) revealed substantial differences in the genetic stability of individual TBEV genes under the different selection pressures exerted by the invertebrate and vertebrate host environments (Paper II and III). NGS has revolutionized genomic research by providing the comprehensive genomic details of a given
virus population. In **Paper II**, NGS analysis of cDNA-derived infectious clones demonstrated rapid (at passage 0) development of diverse TBEV variants. This observation challenges the conclusions derived from mutation studies based on cDNA-derived viruses (Gritsun *et al.*, 2001; Sumiyoshi *et al.*, 1995; Tajima *et al.*, 2010; Zhao *et al.*, 2005) and emphasizes the importance of using NGS of the rescued viruses to make sure that the observed effects were because of the specific mutation and not due to altered quasispecies structure of the given virus. In **Paper III**, NGS data analysis revealed similarities between the quasispecies populations of two TBEV strains from a natural focus, indicating TBEV persistence in nature. NGS of additional TBEV strains from ticks or rodents sampled at natural foci will strengthen our understanding of quasispecies structure and the perseverance of TBEV in nature.
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امی اور ابومی کے نام

دہوندی نا ربا کوئی لفظ جو کر سکے حقی شکریہ ادا

جا کر تُہری نگاه تو نظر آیا الحمَّدِ اللَّه

نويدَ
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TICKS AND TICK-BORNE ENCEPHALITIS VIRUS


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Tick-borne encephalitis (TBE) is a vector-borne disease with severe neurological outcomes. The causative agent, TBE virus (TBEV) is usually transmitted to humans through tick-bite. Environmental factors play an important role in TBE distribution by regulating interactions between ticks and humans. Climate change has contributed to increased tick abundance and incidence of tick-borne diseases, and between 10,000 and 15,000 human TBE cases are reported annually in Europe and Asia. Naveed Asghar has studied the probability of seasonal synchrony of questing ticks and total tick abundance, which are important factors for persistence of TBEV in nature. He has identified TBEV quasispecies within the virus pools from cell culture, infected mice brain, questing- and blood-feeding ticks. His data indicate that the selection pressure faced by the TBEV quasispecies in different hosts affects the virus quasispecies evolution within respective host. He has also investigated the putative role of quasispecies and the 3’ non-coding region in TBEV virulence. In addition, he identified similarities between the TBEV quasispecies pools of two TBEV strains from a natural focus, indicating TBEV persistence in nature.

Naveed Asghar graduated with a Bachelor of Pharmacy degree from University of the Punjab, Lahore, Pakistan in 2008. He was awarded a Master of Science degree in Molecular Biology from Södertörn University, Sweden in 2010. He carried out his PhD studies at Södertörn University within the field of Environmental Sciences with a special focus on the Tick-borne encephalitis virus. His thesis work was also performed at Umeå University, Sweden and Örebro University, Sweden where he combined the research with teaching at School of Medical Sciences.

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Ticks and Tick-borne Encephalitis Virus
From nature to infection

Naveed Asghar

SÖDERTÖRN DOCTORAL DISSERTATIONS