Epidemiological and Ecological Studies of Tick-borne Encephalitis Virus

Pontus Lindblom
About the cover

The cover displays a nymph of the tick species *Ixodes ricinus*.

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Intelligence
Not because you think you know everything without questioning,
but rather because you question everything you think you know
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Abstract

Ticks are blood-sucking parasites that are an inconvenience for both humans and animals. The tick by itself is normally harmless unless they attack in excessive numbers. The harm from ticks stems from them being excellent vectors for other parasites, in the form of bacteria and virus that via the ticks are provided a bridge to move across the blood streams of different animals, including humans.

One of the most pathogenic tick-borne disease for humans is caused by a flavivirus, the tick-borne encephalitis virus (TBEV). Each year approximately 10,000 individuals on the Eurasian continent develop neurological disease, in the form of meningitis, encephalitis, myelitis and radiculitis, following a bite by a TBEV infected tick.

To evaluate the risk of TBEV infection after a tick-bite, we have developed a study to investigate ticks that have bitten humans and to follow up the tick-bitten humans to investigate if they get infected, and if they develop symptoms, and further trace the virus back to the tick that is infected with TBEV. Ticks, blood samples, and questionnaires were collected in collaboration with 34 primary health care centers in Sweden and on the Åland Islands during 2008 and 2009.

Several demographical and biological factors were investigated regarding the interaction between ticks and humans. The main finding was that men removed the ticks later than women, and that both older men and older women removed the ticks later than younger individuals. This could in part explain why older individuals in general, and men in particular, are at greater risk of acquiring tick-borne encephalitis (TBE).

Furthermore, the prevalence of TBEV in ticks that have bitten humans were investigated, in order to correlate the copy number of TBEV in the tick and the tick feeding-time to the risk of developing symptomatic and asymptomatic infection. This entailed the development of new methodology for tick analysis and TBEV real-time PCR. The result showed a very low risk of TBEV infection in the studied areas, only 5 of 2167 investigated ticks contained TBEV. Three of the individuals bitten by TBEV infected ticks were vaccinated and did not develop symptoms of TBEV infection. One unvaccinated individual got bitten by a tick containing 1800 virus copies, with a feeding-time of 12-24h, and interestingly showed no signs of infection. Another unvaccinated individual got bitten by a tick containing 7.7 million virus copies, with a feeding-time of >60h. This individual developed symptoms consistent with a 1st phase of TBE, including fever and headache, but did not develop the 2nd neurological phase of TBEV infection. Despite only finding 5 ticks infected with TBEV, a correlation between the virus load in the tick and the tick feeding-time was observed. In 2 other individuals, TBEV antibody seroconversion was detected during
the 3 month study period, one without symptoms, while the other experienced symptoms consistent with the 1st phase of TBE. These observations support the hypothesis that a higher virus amount in the tick and a longer feeding time increases the risk of TBEV infection.

To further examine TBEV in ticks that have bitten humans and find factors that may predict the risk of human infection and development of TBE, we characterized several TBEV strains genetically. Including TBEV strains isolated from ticks that have bitten human, from questing field-collected ticks, and TBEV strains isolated from patients with TBE. In one of the ticks detached from a human after >60h of feeding, there was a heterogeneous population of TBEV quasispecies with varying poly(A) length in the 3’ untranslated region of the genome was observed. These variations might have implications for differences in virulence between TBEV strains, and the heterogeneous quasispecies population observed could be the virus adapting from replication in tick cells to mammalian cells.

We also investigated the response to TBEV vaccination in relation to 14 health-related factors in a population of older individuals on the Åland Islands. Blood samples, questionnaires, and vaccination records were collected from 533 individuals. Three different serological assays to characterize antibody response to TBEV vaccination were used. The main finding was that the number of vaccine doses in relation to age was the most important factor determining successful vaccination. The response to each vaccination dose declined linearly with age, and as much as 47% of individuals 50 years or older that had taken 3 vaccine doses were seronegative, compared to 23% that had taken 4 doses and 6% with 5 doses. Comparison between the serological assays revealed that the cutoffs determining the balance between sensitivity and specificity differed, but not the overall accuracy.

Taken together, these results contribute to a better understanding of the TBEV epidemiology and can provide tools in the prevention of TBE.
Populärvetenskaplig sammanfattning


Vi har därför skapat en vetenskaplig studie, kallad "STING-studien", där vi undersöker fästingar som har bitit människor för att se om de bär på viruset. Vi följer de fästingbitna personerna och ser om de utvecklar antikroppar som tecken på TBE-virus infektion, samt för att se om de blir sjuka efter fästingbettet. Under 2008 och 2009 deltog 1886 fästingbitna personer som det samlades in fästingar, blodprover och enkäter ifrån i samarbete med 34 vårdcentraler belägna i södra Sverige, mellersta Sverige, Umeå och Åland.

Vi analyserade hur, var och när människor blir fästingbitna, samt hur länge fästingen suger blod innan den upptäcks och plockas bort. Resultatet visade att män plockar bort fästingar senare än kvinnor och att det tar längre tid för både äldre kvinnor och äldre män att upptäcka och plocka bort fästingen. Ju längre tid fästingen får suga blod desto större risk är det sannolikt att virus överförs. Det skulle delvis kunna förklara den högre observerade risken för äldre personer i allmänhet och män i synnerhet att drabbas av TBE.

Vi analyserade även förekomsten av TBE-virus i fästingarna som bitit människor. Viruset hittades endast i 5 av 2167 analyserade fästingar, vilket indikerar att risken att bli biten av en fästing som bär på TBE-virus i de undersökta områdena är väldigt låg. Tre av dessa personer blev bitna på Åland och de var alla vaccinerade och insjuknade inte i TBE. Två personer var inte vaccinerade. Den ena personen blev biten nära Uppsala av en fästing som sugit blod 2 - 3 dygn och innehöll 7,7 miljoner TBE-virus. Trots det fick personen endast feber och huvudvärk i 4-5 dagar innan tillfrisknande. Den andra personen blev biten i Kalmarsen av en fästing som sugit blod 12-24h och innehöll 1800 TBE-virus. Denna person blev varken sjuk eller utvecklade antikroppar som tecken på att viruset överförts vid fästingbettet. När vi sedan undersökte blodet från alla fästingbitna så hittades 2 personer som utvecklade antikroppar mot viruset under studietiden men
där virus inte kunde hittas i någon av de fästingar som lämnats in till vår studie. En av dessa personer rapporterade huvudvärk, nackstelhet och feber men tillfrisknade sedan och den andra hade inte haft några symtom. Sammantaget ger dessa olika förlopp stöd för uppfattningen att många viruskopior i fästingen och lång blodsugningstid ökar risken att bli infekterad.

För att hitta ytterligare faktorer som kan påverka risken att infekteras och utveckla TBE så undersökte vi genetiska skillnader mellan olika TBE-virus. Vi karaktäriserade delar av arvsmassan på virusstammar från fästingar som sugit blod från människor, från fästingar som insamlats i fält och TBE-virus från patienter med TBE. I en av fästingarna som sugit blod från en människa 2 – 3 dygn så upptäcktes variationer i en del av arvsmassan. Hypotesen är att detta beror på att vi fångat fästingen just i den stund då de virus den innehåller håller på att förändras från att vara anpassade till at föröka sig i fästingceller till att bli bättre anpassade till att föröka sig i människoceller. Dessa variationer under fästingens blodsugning kan ha betydelse för virusets sjukdomsframkallande förmåga och vara ytterligare en anledning till att ta bort fästingar så snart som möjligt.

För att kunna individanpassa och förbättra preventionen av TBE så undersökes skyddseffekten av TBE-vaccination i förhållande till ålder, kön, antal vaccindoser och 11 andra hälsorelaterade faktorer. Immunsvaret mot vaccineringsvisade sig avta med stigande ålder och äldre personer behöver därför ta fler vaccindoser för att komma upp i samma antikroppsivåer som yngre. Av de deltagare över 50 år som tagit 3 vaccindoser var det bara ca hälften (53%) som hade antikroppar i blodet mot TBE-virus, av de som tagit 4 doser hade 77% antikroppar, och 94% av de som tagit 5 doser.

Dessa resultat är viktiga pusselbitar för att förstå riskerna kopplade till TBE-virus och fästingbett och kan ge nya verktyg för att hindra ökningen av TBE.
List of papers

This thesis is based on the following papers


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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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</thead>
<tbody>
<tr>
<td>C</td>
<td>Capsid (protein)</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>E</td>
<td>Envelope (protein)</td>
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<tr>
<td>EC</td>
<td>External controls</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>ER</td>
<td>Endoplasmatic reticulum</td>
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<td>IC</td>
<td>Internal control</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<td>M</td>
<td>Membrane (protein)</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>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>prM</td>
<td>Precursor to membrane (protein)</td>
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<tr>
<td>RFFIT</td>
<td>Rapid fluorescent focus inhibition test</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>SPR</td>
<td>Serum positivity rate</td>
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<tr>
<td>TBD</td>
<td>Tick-borne diseases</td>
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<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>TBEV-Eu</td>
<td>European subtype of TBEV</td>
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<tr>
<td>TBEV-FE</td>
<td>Far-Eastern subtype of TBEV</td>
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<tr>
<td>TBEV-Sib</td>
<td>Siberian subtype of TBEV</td>
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<tr>
<td>UTR</td>
<td>Untranslated region</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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Introduction

Tick-borne encephalitis virus (TBEV) is a virus transmitted to humans by ticks. The virus can cause severe and sometimes fatal infection of the central nervous system (CNS). One reason humans get ill from TBEV is that we are an accidental host for the virus. TBEV has not coevolved with humans for thousands of years but with other vertebrates and ticks that are its natural hosts. As can be expected with long coevolution, the natural hosts of TBEV do not get ill, or get only mild symptoms. There is also evidence that TBEV changes the behavior of at least its tick host, to increase its chances of transmission to new hosts [1].

Since humans can get seriously ill if infected by TBEV through a tick-bite, and since the number of individuals afflicted by tick-borne encephalitis (TBE) is increasing in Sweden and other countries on the Eurasian continent, it is important to learn more about TBEV and the ticks that transmit the virus. If we can gain a better understanding of the ecology and epidemiology of the ticks and the virus, we are better equipped to mitigate risks they pose to humans.

To acquire more knowledge about human interaction with ticks and the risk for tick-borne diseases (TBDs) after a tick-bite, and also to evaluate the effectiveness of the available vaccine, we conducted a comprehensive study, denoted the TBD STING-study. In this study we recruit tick-bitten humans and investigate the pathogen content in the ticks and follow up the tick-bitten individuals with blood samples and questionnaires to determine if they develop antibodies and symptoms of TBEV infection.

Evolutionary perspectives on parasites and disease

It is possible and in fact most likely that the great majority of species on earth are parasites, and that the “free-living” species that have been the main focus of almost all ecological and biological studies to date are the minority of life-forms on earth [2,3]. Parasites exert a powerful evolutionary force, because of their abundance and direct predation on other species, which gives rise to adaptations to combat the intruder through natural selection and coevolution [4]. The most widely accepted definition of a parasite is an organism which lives inside or on the surface of another species (the host) from which it extracts nutrients to the detriment of the host [5]. Parasites are generally smaller than their hosts and have a shorter generation time. Parasites that live on the surface of a host are classified as ectoparasites (e.g. ticks, fleas, lice and
mites), and those that live inside the host are called endoparasites. Intercellular endoparasites live in the spaces and fluid between the cells of a host organism (e.g. parasitic worms and certain bacteria species), whereas intracellular endoparasites live inside cells of the host organism (e.g. viruses and certain bacteria, fungi and protozoa species). Traditionally bacteria and viruses were not classified as parasites but this dogma has changed and now parasites can range from sequences of nucleic acids, viruses, and bacteria to plants, fungi and animals [6].

Viruses have likely existed on earth as long as the earliest cells, and they are abundant in all forms of life, from animals and plants to bacteria and archaea [7]. Viruses are genetic elements (RNA/DNA) enclosed in a protein capsule, which code for the ability to spread between and replicate in living cells. Due to this mechanism of horizontal gene transfer between different cells, viruses play a very important role in evolution [8]. A virus has no metabolism and is therefore dependent on a cell for its replication, i.e. virus by itself can neither break down organic matter to harvest energy (catabolism), nor use energy to synthesize larger molecules from smaller subunits (anabolism). Only cells have this ability and this is what makes them able to build ordered structures from the environment and replicate independently, that which most would define as the distinguishing feature of life. Metabolism is the unique feature of life which allows cells to increase its order and complexity locally by decreasing the order and complexity of its surrounding environment. By this definition a virus is not alive but rather an inanimate object that through its molecular structure is interpreted by cells in a way that the cell machinery is instructed to create new copies of the virus. The range of cell types and host species a virus can infect is called the host range. Viruses have evolved different ways of spreading between susceptible host cells. A common way include the oral-fecal route, where viruses have adapted to endure long exposures to different environmental conditions. Other viruses have adapted to be transported directly between the blood streams of host animals through a third organism called a vector. Arthropod blood parasites such as mosquitos, flies, lice, fleas and ticks are important vectors for the spread of many viruses, bacteria and other small parasites.

An organism can be described in the words of Richard Dawkins as "an entity all of whose genes share the same stochastic expectation of the distant future" [9]. The genes and the phenotype they express regardless of what organism they are part of can be seen as the true competitors in natural selection, and the reason why genes are seen to cooperate within an organism is because they share the same exit route into the future [9]. However, a parasite within an organism that do not share the same exit route to the genetic future will evolve to change the host phenotype in a direction that is beneficial for its own propagation, often to the detriment of the host. Parasites that can travel from host to host within the same generation (horizontally), are the kinds of parasites that gives rise to diseases. A disease is a phenotypic expression of either the parasites genes in the host to benefit the parasites reproduction, or the side effects of
the hosts attempt to combat the parasite. However, if an individual parasite lose the ability to spread horizontally and instead gains the ability to spread from host to offspring (vertically), it will be stuck with the same genetic vehicle of propagation as its host, and will with time merge with the host and become benign and even give evolutionary advantages in order to maximize the chances of procreation. The mitochondria and chloroplasts are believed to have originated in this way [10]. There is now strong evidence that infection with viruses sparked the evolution of placental mammals, by providing a protein that allows cells to fuse together [11]. At least 8% of the human genome is made up of viral genes, and 45% is made up of other mobile genetic elements that have integrated themselves in the genome in a coevolution over millions of years [11].

The Tick

Ticks (order: Ixodida) are ectoparasites that diverged from spiders, scorpions and mites several hundred million years ago [12]. Ticks obtain nutrients by ingesting blood from a wide range of mammals, birds and lizards [13]. There are >700 species of hard ticks (Ixodidae) and >190 species of soft ticks (Argasidae). Hard ticks are defined by a dorsal shield (scutum), which the soft ticks lack. Ticks are second only to mosquitoes as vectors for transmitting human pathogens [14]. When the tick feeds, its salivary glands function as kidneys that return water to the host and in doing so transmission of pathogens that are located in the saliva and salivary glands can occur [15]. Ticks are sensitive to dehydration and they maintain their water balance by living in the sheltered microenvironment in the grass, moss and leaf litter on the ground floor of forests, fields and meadows. Hot dry summers and cold dry winters without snow cover are most harmful for the tick populations. The distribution of ticks depends on suitable vegetation biotopes, temperature, humidity and the available range of suitable host vertebrates. Ticks of the genus *Ixodes* have no eyes but with sensory organs on their front pair of legs (Haller’s organ) they can sense carbon dioxide, temperature, odors, and movement [16]. *Ixodes* ticks can serve as both reservoir hosts and vectors for TBEV [17].

Life cycle

The life cycle of *Ixodes* ticks consists of 3 stages; larvae, nymph and adult. Each life stage lasts about 1 year, and for each stage the tick needs a blood meal on a new vertebrate host in order to develop into the next stage (Fig. 1). Larvae and nymphs generally feed on smaller mammals
and birds, while the adult female feed on larger animals and then lays 1000 - 5000 eggs [15]. The male adult tick can attach and feed briefly or not at all before mating with a female tick [18].

**Figure 1.** Tick life cycle and the relative importance of different host animals for the life stages. (Courtesy of Professor Jeremy Gray)

The larvae is 0.5 – 0.8 mm, the nymphs 0.9 – 1.2 mm, the male adults 1.8 – 2.1 mm, and adult females 2.1 – 2.6 mm in size when unengorged (Fig. 2). During feeding the body mass of the tick can expand 10 – 200 times. Larvae feed for 3 – 4 days, nymphs 3 – 5 days, and adult females feed 6 – 10 days on average. *Ixodes* ticks quest passively by climbing on to the vegetation at a suitable height for their preferred hosts (<30 cm larvae, <1 m nymphs, <1.5 m adults) and then
wait for a host to brush against them that they can climb onto [15]. Most ticks are found next to wild animal trails. The ticks become active at temperatures above 4 – 5 °C [19]. Periods of peak tick activity vary depending on the climate. In central Europe there is generally a 1st peak in May/June and a 2nd peak in September/October, while in colder regions there is only 1 peak in the summer [20].

Figure 2. Pictures and relative size of unfed: larvae, nymph, adult male, and adult female of *Ixodes ricinus*.

**The virus**

TBEV is a member of the genus Flavivirus, family Flaviridae. Flaviviruses are round, enveloped particles, 40 – 60 nm in diameter. Depending on the vector used for virus transmission, they are divided into 3 groups: mosquito-borne (e.g. Yellow fever virus, Japanese encephalitis virus, Dengue viruses and West Nile virus), tick-borne (e.g. TBEV and Louping ill virus), and viruses with no known vector [21].

Flaviviruses have a positive sense, single stranded RNA genome, about 11 kb in length, which is capped in the 5’ end [22]. The genome encodes a single open reading frame flanked by highly structured 5' and 3' untranslated regions (UTRs) [23]. The 5' UTR is about 100 nucleotides long and the 3' UTR between 350 – 700 nucleotides. Unlike cellular mRNA, the flavivirus lacks a poly(A) tail, and the 3'UTR tail forms a stem-loop structure important for replication [24].

open reading frame translates into a polyprotein approximately 3400 amino acids long (Fig. 3 A), which is cleaved into 3 structural proteins; C (capsid), prM (precursor to the membrane protein), E (envelope) and 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), that are required for replication in the host cell [25]. The genome is enclosed in a nucleocapsid made of protein C, which is surrounded by a membrane consisting of proteins E and M (Fig. 3 B). The E protein is the major surface glycoprotein responsible for receptor mediated endocytosis and membrane fusion [26,27]. The E protein is the most important determinant of viral host range, tissue tropism, virulence and induction of protective immunity [28].
Replication

The viral life cycle (Fig. 3C) begins by virus entry into the host cell via receptor-mediated endocytosis using the clathrin-mediated pathway [30]. The receptor that mediates entry into the host cell is unknown, but the glycosaminoglycan heparan sulphate, which is abundantly expressed on many cell types, including those in vertebrate and tick tissues, seems to be important for virus attachment [31]. The virus-containing vesicle fuses with endosomes and proceeds down an endosomal and lysosomal endocytic pathway [32]. A pH-triggered conformational rearrangement of protein E, from dimers to trimers, occurs in the acidic environment of these vesicles at a threshold of about pH 6.6 [33]. This causes the viral membrane to fuse with the endosomal membrane and the viral nucleocapsid is released into the cytoplasm of the host cell. The capsid protein and the viral RNA dissociates in the cytoplasm, and viral RNA is translated on host cells ribosomes, yielding viral polyproteins, which are then cleaved by viral serine protease and cellular proteases into individual proteins [25]. Viral RNA synthesis of negative strand copies, which serve as templates for the synthesis of new positive strand RNAs occurs inside vesicle packets in the cellular membrane [23]. The 3'UTR and 5'UTR of the RNA contains complementary sequences that cause cyclization, needed for binding to the NS5 RNA dependent RNA polymerase and initiation of minus strand RNA synthesis [23]. The prM and E proteins are meanwhile translocated into the lumen of the endoplasmatic reticulum (ER) [34]. Newly synthesized RNA associates with the C protein and is packed into nucleocapsids that buds into the ER lumen, thereby acquiring the lipid bilayer and the E and prM proteins to form immature viral particles [35]. The prM and E protein can also spontaneously associate to form sub viral particles that lack the capsid and viral RNA, making these particles non-infectious [36]. Immature viral particles are transported through the secretory pathway, where final maturation of the virus takes place in the acidic vesicles of the late trans-golgi network, in which protein prM is cleaved by the host cell protease furin [37]. This activates the membrane fusion capability of protein E and is essential for virus infectivity [38]. Transport vesicles fuse with the plasma membrane of the host cell and release the virus through exocytosis.

Subtype distribution

The TBEV virus complex is believed to have evolved during the last 3 – 5 thousand years and have gradually spread toward the west across Asia and Europe [39]. However, this unidirectional spread of the virus has been challenged recently [40,41], together with the evolutionary age of the tick-borne flaviviruses, which has been suggested to be 4 to 6 times older than previously described [42].
TBEV has been divided into 3 distinct subtypes based on the amino acid sequence of the E protein [43,44]. The European subtype (TBEV-Eu) is primarily transmitted by the tick species *Ixodes ricinus* which is common in Europe and the west part of Russia, while the Far-Eastern (TBEV-FE) and Siberian (TBEV-Sib) subtypes are primarily transmitted by *Ixodes persulcatus*, which is common from the Baltic countries all the way across Asia to Japan [43] (Fig. 4). All 3 TBEV subtypes can co-circulate in areas where both tick species overlap [45–47]. Phylogenetic studies have shown that TBEV-Fe and TBEV-Sib are more closely related to each other than to TBEV-Eu, and that TBEV-Eu is more closely related to Louping ill virus, which is the only tick-borne flavivirus in the United Kingdom. Louping ill virus is also transmitted by *I. ricinus* and can cause disease in sheep and red grouse [48].

![Figure 4](image-url)  
*Figure 4. Distribution of the tick species *I. ricinus* that can transmit TBEV-Eu, and of *I. persulcatus* that can transmit the TBEV-FE and TBEV-Sib. The dotted line marks the area in which TBEV can be present. (Reprinted with permission [49])*

To further differentiate TBEV strains into smaller groups for investigation of their origin, distribution, and evolution of the virus, the term “clusteron” has been proposed for the smallest unit of phylogenetic group classification, based on the amino acid sequence of the E protein [50]. Clusteron networks of TBEV-Sib has been shown to exhibit a more complex structure than those of TBEV-FE and TBEV-Eu, and the TBEV-Sib clusterons have a more distinct geographical distribution [50]. This supports phylogenetic analysis suggesting that TBEV originated in central
Russia, with the Siberian lineage being the first to diverge, followed by the European subtype evolving from spread to the west, and the Far-Eastern subtype from spread to the east [40]. The Siberian subtype can be distinctly divided in two groups geographically by single signature amino acids in the E protein, separating Baltic TBEV strains (Finland, Estonia, Latvia) from Siberian (Novosibirsk, Tomsk, Irkutsk) [51].

**Quasispecies**

Despite being a RNA virus, TBEV is genetically stable due to constraints by both tick and vertebrate hosts in its life cycle [52]. However, it has been observed that the virus can change both genetically and phenotypically between propagation cycles in ticks and mice [53,54]. It appears that tick-adapted strains need to propagate in mice a number of times to become pathogenic to mice [55]. Further, TBEV-passaging in cell culture and/or mouse brain can result in spontaneous genomic deletions and elongations within the variable part of the 3’UTR [56,57].

The adaption of TBEV to replicate in tick and vertebrate hosts could be based on a heterogeneous quasispecies population, with a changing ratio of variants when the virus changes environment between different types of host cells, together with new emerging mutants that may promote the adaptation [54].

**Vector-host transmission**

Transmission of TBEV relies on the interaction between virus, ticks, and vertebrates hosting the ticks [58]. TBEV is mainly transmitted from infected to non-infected ticks that are co-feeding on the same host. This may occur without viraemia in the host animal [17,59–61]. Transmission can also occur in animals with immunity against TBEV [62]. A tick that is infected with TBEV carries the virus for the rest of its life [63]. Rodents such as the yellow necked mice (*Apodemus flavicollis*) and the bank vole (*Myodes glareolus*; formerly *Clethrionomys glareolus*) are considered to be most important for TBEV circulation, as they have been shown to support effective transmission of TBEV between co-feeding ticks [17]. Increased size of the rodent population has been connected to an increase in both ticks and TBDs in humans within 1 - 2 years [15]. How TBEV is transferred via Langerhans cells and neutrophils from infected to non-infected ticks, co-feeding on non-viraemic and non-systemically infected animals, has been demonstrated experimentally, and also that skin localized amplification of TBEV occurs in the macrophages [60]. Virus transmission is enhanced by immunomodulatory factors present in the
tick saliva [64]. With experimentally TBEV-infected ticks, virus transmission has been shown to occur within a few minutes of feeding [65].

For TBEV transmission via co-feeding between nymphs and larvae it is important that their seasonal activity coincide [66]. This has been shown to rely on the rate of temperature increase during the spring, where a higher temperature increase rate promotes greater synchrony of nymph and larvae feeding activity [67], which appears to determine the focal distribution of TBEV [68–71]. Non-viraemic transmission of TBEV through co-feeding is believed to be most important for virus circulation, which implies that the ticks most likely are the main reservoirs for TBEV, and that the vertebrates acts as the “transient bridge” for virus transmission from tick to tick, while providing blood-meals for ticks [17,72].

TBEV infection can persist in rodents for several months and even throughout the years, but it is unclear how much this contributes to the transmission and overwintering of the virus [73–76]. Even vertical viral transmission have been shown to occur with the Siberian subtype of TBEV in red voles (Myodes rutilus Pallas) [77].

Transovarial transmission of TBEV can occur from the tick female to the egg (larvae) at a low rate (< 0.5%), which may have an important role in maintaining TBEV circulation [78]. It has been suggested that mass co-feeding of larvae could be important for persistent circulation of TBEV [79].

The role of birds for maintaining TBEV circulation has since long been indicated by presence of antibodies against TBEV in birds [80]. The first study to investigate TBEV RNA in ticks from migrating birds was done on 13,260 migrating birds on the southeast coast of Sweden in 2001, showing that 3.6% of the birds were tick-infested and TBEV RNA was detected in 4 of 1,155 investigated ticks (0.3%) [81]. A study on migrating birds in Western Estonia found that 1 of 249 investigated ticks (0.4%) were infected with TBEV [82]. Despite this low TBEV prevalence, migrating birds may be very important for spreading of TBEV over larger distances, considering that several hundred million birds migrate through Scandinavia every spring and fall [81]. A recent study from a highly TBE endemic region in Western Siberia found TBEV RNA and TBEV antigen in 14.1% of I. persulcatus, in 5.2% of Ixodes pavlovskyi, and 4.2% of Ixodes plumbeus ticks collected from wild birds, in which also TBEV RNA and TBEV antigens were detected in 9.7% and 22.8% of the birds respectively [83]. Although it has never been demonstrated that birds can introduce new tick species or new tick-borne pathogens to new geographical areas, it might explain the discontinuous distribution observed for TBEV [84]. The importance of birds, and if some bird species can serve as reservoir hosts for TBEV, is still unclear.
Some animals may not support transmission of the virus through co-feeding but can be very important in supporting the circulation of TBEV by enabling the tick population to grow large [85,86]. The population dynamics of *I. ricinus* and *I. persulcatus* are highly dependent on the availability of larger animals to support the adult stages [15]. Roe deers (*Capreolus capreolus*) are among the most important hosts for *I. ricinus* and support feeding by both larvae, nymphs and adult ticks. The frequency of co-feeding on roe deer has been shown to correlate with the number of human TBE cases in the north-eastern Italian Alps [71]. It has not yet been verified if roe deer, goats, sheep, cattle and many other important tick hosts can support non-viraemic transmission through co-feeding [58], although it has been shown that they develop a strong antibody response to TBEV, which makes them useful as sentinels for determining TBEV risk areas [87,88].

In the countries of Northern Europe *I. ricinus* accounts for almost all tick infestations on humans, dogs, cats, horses, cattle and deer [13], and the tick has been found on >300 species of wild and domestic mammals, birds and reptiles [89]. In Europe, a marginal presence of TBEV has also been observed in 6 other tick species; *Ixodes hexagonus*, *Ixodes arboricola*, *Haemaphylis concinna*, *Dermacentor marginatus*, and *Dermacentor reticulates* [15]. In Russia, TBEV is spread primarily by *I. persulcatus* ticks [83], while in China TBEV is spread by *I. persulcatus* in the north but by *Ixodes ovatus* in the south [90]. Humans are accidental host for TBEV and do not contribute to sustain either the virus or the tick population [15].

**Prevalence in ticks**

The prevalence of TBEV in ticks has mainly been investigated in questing unfed ticks in the field. Only a few studies have investigated TBEV prevalence in ticks detached from humans. The median TBEV prevalence in field collected ticks is about 0.4%, but varies significantly depending on how targeted the collection of ticks is on areas where individuals that have contracted TBE reported to be tick-bitten (Table 1). In a recent Swedish study, the average prevalence of TBEV in field collected ticks was 0.23%, with a lower prevalence in nymphs (0.1%), than in adults (0.55%), but in a well-known highly endemic Island in the Stockholm archipelago the prevalence was 0.5% in nymphs and 4.5% in adults [91]. In 3 German studies, a higher prevalence of TBEV has been observed in ticks detached from humans than in field-collected ticks from the same area (Table 1) [92–94]. Only a small proportion of infected ticks, both from the field and from humans, appears to have a high virus titer [95,96]. How TBEV can persist at such low prevalence rates in ticks is not completely understood, but mathematical models suggests that aggregation of co-feeding ticks on host animals is crucial to maintain virus circulation, and may explain the highly focal and patchy distribution of TBEV [97].
Table 1. TBEV prevalence in *Ixodes* ticks collected in the field, and ticks detached from humans (Modified from supplementary table S1 in paper II).

<table>
<thead>
<tr>
<th>Country</th>
<th>Year</th>
<th>Prevalence %</th>
<th>Ticks tested</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Field-collected ticks:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Austria</td>
<td>1990</td>
<td>0.4</td>
<td>3,404</td>
<td>nRT-PCR</td>
<td>[98]</td>
</tr>
<tr>
<td>Czech Republic</td>
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<td>0.4</td>
<td>491N</td>
<td>nRT-PCR</td>
<td>[99]</td>
</tr>
<tr>
<td>Denmark</td>
<td>1999</td>
<td>0.05</td>
<td>4,058</td>
<td>nRT-PCR</td>
<td>[100]</td>
</tr>
<tr>
<td>Denmark</td>
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<td>0.2</td>
<td>448</td>
<td>Real-time PCR</td>
<td>[101]</td>
</tr>
<tr>
<td>Denmark</td>
<td>2011</td>
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<td>2,640</td>
<td>Real-time PCR</td>
<td>[102]</td>
</tr>
<tr>
<td>Estonia</td>
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<td>1,770</td>
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<tr>
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<td>Germany</td>
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<td>nRT-PCR</td>
<td>[109]</td>
</tr>
<tr>
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<td>820N, 90A</td>
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</tr>
<tr>
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<td>nRT-PCR</td>
<td>[113]</td>
</tr>
<tr>
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<td>nRT-PCR</td>
<td>[70]</td>
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<td>Italy</td>
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<td>1.2</td>
<td>1,739</td>
<td>Real-time PCR</td>
<td>[114]</td>
</tr>
<tr>
<td>Latvia</td>
<td>2000</td>
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<td>175N, 350A</td>
<td>nRT-PCR</td>
<td>[115]</td>
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<tr>
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<td>0.4</td>
<td>5,630</td>
<td>Real-time PCR</td>
<td>[118]</td>
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<tr>
<td>Poland</td>
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<td>1.6</td>
<td>875</td>
<td>nRT-PCR</td>
<td>[119]</td>
</tr>
<tr>
<td>Russia</td>
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<td>468</td>
<td>Real-time PCR</td>
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<td>4,777</td>
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<tr>
<td>South Korea</td>
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<td>[123]</td>
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<td>Sweden</td>
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<td>Real-time PCR</td>
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<td>9,868</td>
<td>Real-time PCR</td>
<td>[127]</td>
</tr>
</tbody>
</table>

**Ticks from humans:**

<table>
<thead>
<tr>
<th>Country</th>
<th>Year</th>
<th>Prevalence %</th>
<th>Ticks tested</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
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<td>2001</td>
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<td>86N, 129A</td>
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<td>[92]</td>
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<td>1.3</td>
<td>239</td>
<td>Real-time PCR</td>
<td>[94]</td>
</tr>
</tbody>
</table>

N: Nymphs, A: Adults, nRT: nested reverse transcriptase
The disease

History and present epidemiological situation

TBE was first described in 1931 by the Austrian physician Hans Schneider, who observed a seasonal relationship between similar cases of meningoencephalitis, although the viral cause behind the disease and the connection to ticks was not known at the time [128]. The connection to ticks and the discovery of virus as the causative agent was first reported in 1937 during a large expedition in the Far-East of Russia. During this expedition, led by the Soviet scientist Levkovich Zilber [129], the virus was isolated and serologically characterized in the tick vector *I. persulcatus*, reservoir animals, as well as in lethal human cases.

In Europe, TBEV was first isolated from a human TBE patient in 1948 in Czechoslovakia [130]. The following 10-year period, serologically verified TBE cases were reported from Hungary, Slovenia, Austria, Poland, Sweden, and Finland [131].

TBE is endemic on the Eurasian continent from the Balkan Peninsula in the south-east to Scandinavia in the north, and from eastern France in the west throughout central Eurasia to the Japanese Islands in the east [132]. The virus is present in natural foci that can range from a few square meters to many square kilometers [15].

From 1974 to 2003 the number of TBE cases in Europe increased by 400% [133]. Between 1990 and 2007 an average of 8755 TBE cases per year was reported in Europe and Russia, compared to an average of 2755 cases per year between 1976 and 1989 [133]. In the 1990s there was a dramatic increase in TBE cases in eastern Europe [134–136].

This increase may be due to an expanding tick-population promoted by climate change and changes in host animal availability, but also due to social and political changes, as well as improved surveillance, diagnosis, and reporting of TBE cases. Which factors that contribute the most can differ geographically and is often hard to quantify. At the borders of the current TBEV distribution, climate change may have a role, while changes in TBE cases within core endemic areas appears better explained by socio-economic factors [137–152]. In the Baltic countries, increased risk of TBE has been correlated to socioeconomic factors, where lower educated, unemployed, and retired individuals are more often unvaccinated and visit the forest more frequently for recreation and picking berries and mushrooms [153]. However, socioeconomic factors does not explain the increase of TBE cases observed in countries like Sweden, Finland, German and Italy [133].
The first serologically verified cases of TBE in Sweden were diagnosed in 1954 [131]. Since then, a steady increase has been reported of both TBE cases and new TBEV endemic foci during the last decades [86]. From less than 50 TBE cases per year before the middle of the 1980s to between 200 – 300 cases per year at present (Fig. 5).

On the Åland Islands (Finland), the first cases of meningoencephalitis were described in the 1940s, and the TBEV strain named Kumlinge A52 was isolated in 1959 [154]. The Åland Islands are highly TBE endemic and have had over 300 serologically confirmed TBE-cases since 1959 in a population of less than 30 000 individuals [155].

In Denmark, 8.7% of roe deers has been found seropositive [156]. In Southern Norway TBEV can be considered an emerging pathogen where the first cases were reported in 1998, and now there are 10 – 15 cases per year [84]. However, serological indications that TBEV was present at the Western coast of Norway was reported already in 1973 [157]. In Finland a focus of *I. persulcatus* ticks was discovered in the Kokkola archipelago 2004, carrying TBEV-Sib [104].

In Estonia all 3 subtypes of TBEV co-circulates [158].

![Figure 5. Diagnosed TBE cases in Sweden from 1956 to 2013. (Statistics from the Swedish Institute for Infectious Disease Control)](image-url)
**Pathogenesis**

Infection by TBEV occurs primarily through a bite of an infected *Ixodes* tick, although another route of infection is through consumption of non-pasteurized milk from viraemic livestock (goat, sheep, or cow) [159].

Initial viral replication occurs at the local inoculation site of the tick-bite, where TBEV has been demonstrated in immune cells in the skin, e.g. Langerhans cells, neutrophils, and macrophages [60]. Through Langerhans cells, the virus reaches the regional draining lymph nodes via the lymphatic system, where further virus replication takes place. During the resulting viraemia the virus spreads through the blood and lymphatic system, and invades many extraneural tissues, primarily spleen, liver and bone marrow [159]. High levels of virus replication in the primarily infected cells and organs appears necessary for the virus to cross the blood-brain barrier, which thereby influences the neuroinvasiveness [160]. The mechanism for how TBEV invades the CNS is unknown. It may involve passive diffusion over a leaky blood-brain barrier, infection of olfactory neurons, or infection of vascular endothelial cells of brain capillaries, followed by transcytosis, and release of virus into the brain [159].

Entry into CNS is followed by viral replication primarily in the neurons [161]. In mice, the inflammatory reaction mediated by CD8+ T cells significantly contributes to the neural damage, demonstrated by significantly longer survival time by immunodeficient mice [162]. The majority of cells recruited to the CNS during early stages of TBE in humans are T cells, and to a lesser extent B cells and NK cells [163]. Elevated levels of neopterin in CSF from TBE patients compared to aseptic cases of meningoencephalitis suggests that a long-lasting inflammatory response is of pathophysiological significance in TBE [164]. In fatal cases of human TBE, the TBEV primarily targets large neurons of the anterior horns in the spinal cord, the brain stem, cerebellum and the basal ganglia [165].

The cellular chemokine receptor CCR5, expressed mainly on subsets of monocytes, macrophages, NK cells, and T cells, has been shown to be critically important in mouse models of West Nile virus infection, where CCR5⁻/⁻ mice have impaired traffic of white blood cells to the CNS and rapidly dies compared to wild type mice [166]. The CCR5 chemokine receptor is also expressed on neurons, astrocytes and microglia [167]. A study on patients with TBE in Lithuania found a slightly higher CCR5Delta32 allele frequency and homozygous frequency in TBE patients compared to control groups of aseptic meningoencephalitis and matched healthy controls [168]. Further studies are needed to clarify the role of CCR5 in TBE and other neurotropic flavivirus infections such as Yellow fever.
Clinical picture

Serological surveys suggest that between 75 – 95% of TBEV infections in Europe are subclinical [21]. This also appears to be the case in western Siberia where asymptomatic infection is most common, and of the symptomatic infections, between 60 – 90% manifest in a febrile form without CNS involvement or with only mild transient CNS disturbance [169].

The mortality rate of symptomatic infection is on average less than 1% for the European subtype [170], up to 5 – 20% for the Far-Eastern subtype [171], and 1 – 3% for the Siberian subtype [172].

The Far-Eastern and Siberian TBE generally have a monophasic course for most patients (85 %) [173], while in Europe between 74 to 87% of the patients have a biphasic course of the disease [174,175]. The Siberian and Far-Eastern TBE is commonly observed in children [176], who experience a higher frequency of the severe forms of meningoencephalitis [169]. In contrast, the European TBE rarely affects children and the incidence and severity of TBE increases with age [175,177–179]. There is also more men than women that contract TBE in Europe [174,175,180,181].

For the biphasic clinical course of European TBE (Fig. 6), the median period between tick-bite and onset of the first symptoms is 8 days (range 4 – 28), after which the viraemic 1st phase of TBE appears as an uncharacteristic influenza like illness, lasting typically 2 – 4 days (range 1 – 8), during which fever, headache, myalgia, fatigue, gastrointestinal symptoms, decreased number of white blood cells and platelets in the blood commonly occur in the viraemic phase [173–175,178]. After an asymptomatic period of median 8 days (range 1 – 33), up to 20 – 30% of the infected patients suffer from a 2nd, meningoencephalitic phase [182]. However, the proportion of patients that develop the 2nd phase of TBE is uncertain and has been challenged by a Slovenian study reporting that all tick-bitten individuals who experienced febrile illness within 6 weeks and developed specific IgM and IgG antibodies to TBEV, also got the 2nd phase of TBE [183].

The 2nd phase of TBE can manifest as inflammation in either the membranes surrounding the brain (meningitis), the brain (encephalitis), the spinal cord (myelitis), or the nerve roots (radiculitis), or a combination of these [159]. TBE presents as a milder form of meningitis in 43 – 55%, moderate meningoencephalitis in 36 – 43%, and severe meningoencephalomyelitis in 8 – 12%, spinal nerve paralysis is present in 5 – 15%, and cranial nerve paralysis in up to 11% of cases [170]. Symptoms of the 2nd phase can include headache, ataxia, altered consciousness, decreased concentration and memory, irritable response to light and sound, dysphasia, dysaesthesia, respiratory insufficiency, tremor and paresis of the extremities [174,175,178].
Laboratory findings during the 2nd phase include specific IgM and IgG antibodies against TBEV in serum and cerebrospinal fluid (CSF). The white blood cell count in CSF is usually only moderately elevated in TBE, with predominance of polynuclear cells initially, which switch to predominance of mononuclear cells after a few days [174]. The ratio of CSF to serum albumin indicates the degree of damage to the blood-brain barrier, which reaches a maximum after a median of 9 days of encephalitis and is observed in 80% of patients [174,178].

Symptomatic TBE-infection often causes permanent damage to the CNS, a condition referred to as post-encephalitic syndrome. Up to 46% of patients at long term follow up have cognitive and neuropsychiatric sequelae [170]. Most common symptoms include e.g. headache (11 – 23%), concentration disturbance (8 – 15%), reduced memory (11 – 20%), tremor (2 – 10%), ataxia (6 – 7%), and paralysis (2 – 6%) [170,174,178].

Figure 6. Biphasic clinical course of European TBE.

**Diagnosis**

Laboratory diagnosis of TBE is primarily established by detection of TBEV-specific IgM antibodies in serum using Enzyme-linked immunosorbent assay (ELISA) [184]. IgM activity in serum is found in 96% of patients a median of 3 days after onset of encephalitis, and in all patients after a median of 9 days [185]. Thereafter, IgM antibody levels slowly decline, but increased levels are
still detected in 33% of patients after 1 year [185]. IgG activity in serum peaks 6 weeks after onset of the disease [185]. Lumbar puncture should be performed to verify CNS infection by Pleocytosis, and detection of intratheal antibodies to exclude differential diagnosis [173]. Intrathecal TBEV antibodies are found in 97% of patients after a median of 9 days [185]. Pleocytosis is often observed in CSF with mononuclear dominance, but not as pronounced as in other viral encephalitis [186]. The ELISA assays are cross-reactive with other flaviviruses, so if the patient has been vaccinated for, or exposed to other flaviviruses (e.g. Yellow fever or Japanese encephalitis), a neutralizing antibody test must be performed for confirmation [184,187].

Viral RNA detection is only possible during the viraemia in the 1st phase of TBE, before development of antibodies [188]. Since most TBE patients do not seek medical care during the 1st phase, PCR and virus isolation is not used for routine diagnosis [186].

Diagnosis of vaccination breakthrough are characterized by higher levels of specific IgG and neutralizing antibodies compared to unvaccinated TBE patients, but a lower level and slow development of specific IgM antibodies [189,190]. To verify vaccination breakthroughs it is necessary to demonstrate production of specific IgM and IgG antibodies in CSF [191].

Treatment

There is currently no specific treatment available for TBE [186]. Symptomatic treatment consists of ensuring sufficient nutrition and water intake, administration of pain killers, mild nonsteroidal anti-inflammatory drugs, and if necessary, antiepileptic drugs [191]. Patients with severe CNS symptoms must be closely monitored as coma and paralysis may develop rapidly in which case artificial breathing assistance is needed [171]. Administration of corticosteroids has been associated with longer hospital stay and worse outcome compared to patient who only receives symptomatic treatment [178]. Treatment by passive immunization with specific immunoglobulins against TBE is no longer available due to lack of evidence regarding the efficacy, and indications that it may aggravate the disease [192]. There is also no evidence to support the use of post-exposure active immunization after a tick-bite [193].

Vaccine

Vaccination is the most important preventive measure against TBE. There are 2 vaccines available in Europe for active immunization against TBEV; FSME-IMMUN (Baxter, Austria), and Encepur (Novartis, Germany) [194]. FSME-IMMUN was introduced on the market in 1976 as the first vaccine for TBEV available in Europe [195], and Encepur was licensed in 1991 [194].
The antigen content is composed of formaldehyde inactivated whole virus of the European TBEV subtype, strain Neudörfl 2.4 µg in FSME-IMMUN (adults) and strain K23 1.5 µg in Encepur (adults), both have children formulas with half the antigen content [196].

In Russia and a few neighboring countries, 2 vaccines based on Far-Eastern TBEV strains are available; TBE Moscow Vaccine (Chumakov Institute, Russia), and EnceVir (Microgen, Russia) [197]. Studies suggest that all 4 vaccines give cross-protection against all 3 subtypes of TBEV [197–199].

In Austria over 600 TBE cases per year was recorded during the 1980s. After the start of a mass vaccination campaign in 1981, the vaccination coverage increased from 6 % in 1980 to 86 % in 2001 leading to an almost 90% decrease in TBE cases [200]. The field effectiveness of the vaccine has been calculated to be 96-99% for regularly vaccinated individuals in Austria 2000-2011, and is estimated to have prevented >4,000 cases of TBE during that time period [179].

The World Health Organization (WHO) recommends vaccination for whole populations in highly endemic areas, defined as >5 cases per 100,000 inhabitants yearly, and vaccination of risk groups in low to moderate endemic areas, defined as <5 cases per 100,000 inhabitants yearly [201].

The conventional primary vaccination schedule for both FSME-IMMUN and Encepur requires 3 doses during the 1st year (months 0, 1, 5-12), and after 3 years a booster dose is needed, and subsequent boosters at intervals of 5 years, or 3 years if ≥60 years [196].

The seroconversion rate has been shown in clinical trials to be close to 100% for adults (16 – 65 years) [202,203], and children (< 16 years) after 3 doses of FSME-IMMUN [204,205], and equal seroconversion rates has been reported in children (< 12 years) and adults vaccinated with the Encepur vaccine [206,207]. The FSME-IMMUN and Encepur vaccines are interchangeable and doses of either vaccine can successfully be followed by the other [208].

Rapid vaccination schedules are available for both vaccines. With the rapid schedule using FSME-IMMUN (days 0, 14, and month 5 –12), the immune response after the 2nd dose is lower than for the conventional schedule, and antibody levels decline more rapidly [207]. Using a rapid schedule with the Encepur vaccine (days 0, 7, 21) in children has been shown to provide fast protection and stable antibody levels for at least 1 year [209]. The antibody response to TBE vaccination declines with age [210–216], and older individuals are at higher risk of vaccine failures [189,200,217]. Both the European vaccines are considered safe to use and efficacious for individuals ≥1 years old by the WHO [201]. Fever is reported in about 20% of children, and in a few percent of adults after the 1st dose [195,201,204,212,218]. There are no published reports on serious adverse effects [219].
Aims

Since humans can get seriously ill if infected by TBEV through a tick-bite, and the number of individuals afflicted by tick-borne encephalitis (TBE) are increasing in Sweden and other countries on the Eurasian continent, it is important to study TBEV and the ticks that transmit the virus. If we can gain a better understanding of the ecology and epidemiology of the ticks and the virus, we are better equipped to mitigate risks they pose to humans.

The general aim of this thesis was to investigate the interaction between ticks, humans and TBE-virus, to increase the knowledge of what factors are most important to prevent TBE.

The specific aims were to investigate:

- How the interactions between ticks and humans regarding tick attachment site, blood-feeding time, and seasonal patterns, affect the risk of contracting TBEV infection.

- How the serological and clinical response of tick-bitten humans relate to viral copy number of TBEV in the tick, tick life stage, and tick feeding time.

- How age, gender and different health related factors affect the chances of obtaining high antibody levels after vaccination against TBEV.

- How TBEV in ticks is affected genetically during tick feeding, and how this may be important for the risk of human infection and virulence of the virus.
Materials and methods

Little is known about the risk of infection and development of symptoms after being bitten by a tick that carries TBEV. To investigate which factors are most important and how they contribute to human TBEV infection and symptom development, the golden standard would be an experimental study with full control of and the ability to vary as many variables as possible. However, it is not ethically possible to let people get bitten by TBEV infected ticks and vary different factors e.g. the tick life stage, strain of TBEV in the tick, quantity of TBEV in the tick, time of blood feeding, position of the tick on the human body, age, gender and health status of the human etc. Instead, the best option we believe is to perform a prospective observational study with voluntary recruitment of humans that get bitten by ticks. Thereby, making it possible to investigate different factors and correlate them to clinical and serological outcome in the tick-bitten individuals. Furthermore, evaluation of TBEV vaccination is possible by measuring antibody titer in vaccinated participants in the study.

In this section the study design, methods and method developments needed for answering our research questions are described, with a brief discussion regarding considerations that emerged during the development of the tick-analysis. More details can be found in the method sections in the respective papers.

The materials and methods used in this thesis are summarized in Table 2 and Table 3.

Table 2. Materials analyzed in each paper.

<table>
<thead>
<tr>
<th>Material</th>
<th>Paper I</th>
<th>Paper II</th>
<th>Paper III</th>
<th>Paper IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ticks that have bitten humans</td>
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<td>2167</td>
<td>3(^b)</td>
<td>2(^b)</td>
</tr>
<tr>
<td>Questionnaires(^a)</td>
<td>1770(^b)</td>
<td>1886</td>
<td>533(^b)</td>
<td></td>
</tr>
<tr>
<td>Serum samples(^a)</td>
<td>1670</td>
<td>533(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaccination records(^a)</td>
<td></td>
<td>494</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBEV strains</td>
<td>5(^*)+12</td>
<td></td>
<td></td>
<td>2(^{a,b})+6</td>
</tr>
</tbody>
</table>

\(^a\)From tick-bitten humans in the TBD STING-study.  
\(^b\)Analysis of subsets of the same material as in paper II.
Table 3. Methods used in each paper.

<table>
<thead>
<tr>
<th>Method</th>
<th>Paper I</th>
<th>Paper II</th>
<th>Paper III</th>
<th>Paper IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tick photo &amp; measurement</td>
<td>X</td>
<td>X</td>
<td>X*</td>
<td>X*</td>
</tr>
<tr>
<td>RNA extraction</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>cDNA synthesis</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Real-time PCR</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nested PCR</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Sequencing</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrosequencing</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In vitro transcription</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Cloning</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Phylogenetic analysis</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Serological analysis</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Questionnaire analysis</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Vaccination record analysis</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

*Described in paper I and paper II.

Ethics

Ethical approval for the TBD STING-study was granted by the Regional Ethics Committee in Linköping (M132-06), and by the local Ethics Committee of the Åland Health Care, 2008-05-23, following principles expressed in the declaration of Helsinki. Each study participant gave their written informed consent before entering the study, and were allowed to discontinue their participation at any time and have their samples discarded. The samples and records were blinded and coded to preserve integrity and anonymity of each participant. The participants were not told the results regarding their individual ticks and blood samples that they provided to the study. However, we have visited the regions from where participants have been recruited several times per year, and given presentations on the results directed to the public. By the results of the STING-study we hope to calm the fears regarding ticks and TBDs by increasing the knowledge of the risks and preventive measures.
The tick-borne diseases STING study

The TBD STING-study started in 2007 and is still ongoing (2014). The aim is to collect 10 000 ticks from tick-bitten humans. Advertisement through local newspapers, radio, television, and informational posters are used to inform people about the study (Fig. 7). Individuals 18 years or older that get tick-bitten can volunteer to participate by bringing the tick(s) that bit them to a participating primary health care center, where they give informed consent, fill in a questionnaire and give a blood sample, and 3 months later they come back for a follow up blood sample and to fill in a 2nd questionnaire.

Figure 7. Schematic depiction of the TBD STING-study design.
The materials included in this thesis was collected in collaboration with 34 primary health care centers during 2008 and 2009 (Fig. 8). We provided prepared test kits that included all the paper work and test tubes needed for each participant. During the 3 month study period the participants were also asked to collect any additional ticks that had bitten them. The ticks and blood samples were sent to Linköping University within 3 days, where they were taken care of and stored in -70 °C until analysis. If a participant developed any symptoms during the study period they were asked to visit the primary health care center for checkup with additional samples and treatment when needed, and all medical records from participants that attended health care due to symptoms possibly related to TBDs were examined in detail by experienced physicians.

![Figure 8](image_url)

**Figure 8.** The location and name of the 34 primary health care centers included in the TBD STING-study 2008 and 2009, from where tick-bitten individuals were recruited. Ystad, Blekinge, Mullsjö, and Åmål only participated in 2009. SE: Sweden, NO: Norway, FI: Finland, DK: Denmark, EE: Estonia, LV: Latvia, LT: Lithuania. (Modified from figure 1 in [220])

A: Southernmost Sweden (507 participants)
Åhus, Hästveda, Knislinge, Ljungdala, Ostby, Perstorp, Vänhem, Vinslöv, Hässleholm, Ystad, Blekinge, Kalmar

B: South-central Sweden (746 participants)
Bankeryd, Gränna, Häbo, Mullsjö, Ätvidaberg, Ekholmen, Johannelund, Kisa, Långem, Linköping, Mjölby, Skärbacka, Söderköping, Vikbolandet, Valdemarsvik, Åmål, Lidköping, Mariestad

C: Northern Sweden (18 participants)
Umeå, Holmsund, Sävar

D: The Åland Islands (615 participants)
Mariehamn
Questionnaires

The participants answered the 1st questionnaire at inclusion in the study and the 2nd questionnaire at the 3 month follow up (appendices in paper I and paper III). The 1st questionnaire contained questions regarding the time point when they discovered the tick, where on the body it was attached, when it was removed, when they think they received the tick-bite, and in what kind of vegetation. Furthermore, they were asked if they had had other tick bites the same season, and if they previously had and been treated for various TBDs. The 1st questionnaire also contained questions concerning the general health status, including asthma, allergy, diabetes, tumors, medication, smoking, vaccinations against TBEV, Yellow fever and Japanese encephalitis, and if they owned pets.

The 2nd questionnaire contained questions regarding additional tick-bites during the study period and general health condition. They were also asked if they experienced any of the following symptoms: headache, fatigue, fever, neck pain, loss of appetite, nausea, weight loss, vertigo, concentration difficulties, radiating pain, muscle or joint pain. Furthermore, how many days symptoms lasted, if they visited health care for the symptoms, and if any reported symptoms appeared before or after any additional tick-bites.

Tick photo and measurement

Pictures and measurements were taken of the tick using a digital USB-microscope. The pictures were used as documentation and to determine life stage, species and general condition of the tick. Measurements were used to determine the feeding-time of the tick using calculation of coxal and scutal indices for the change of body dimensions. This method has been experimentally derived for nymphs and adult female I. ricinus ticks feeding on rabbits [221]. The coxal index gives the best time estimate for ticks that have fed <24h and the scutal index for ticks that have fed >24h (Fig. 9).
Development of RNA extraction from ticks

Individual ticks were placed in 2ml tubes with lysis buffer and a 5 mm steel bead. Batches of 48 samples at the time were homogenised using bead-beating. Each batch consisted of 46 tubes containing ticks, 1 tube containing lysis buffer spiked with TBEV (positive control), and 1 tube contained only lysis buffer (negative control). The lysis buffer serves to free and preserve the RNA in the sample. When developing the method, we aimed to be able to isolate intact TBEV from part of the homogenized tick if TBEV were later found in the PCR. A number of trials were conducted where we varied time, frequency and bead size in the bead-beating process, and we also tried bead-beating the ticks in different liquids, i.e. phosphate buffered saline, and RNA-later, in order to establish a method where intact virus could be preserved for later isolation. Bisecting the tick was ruled out because of the laborious work needed and increased risk for contamination, degradation and uneven handling of the samples. However, it was concluded during the trials that it was not possible to establish a method with the high sensitivity needed for detection and quantification of tick-borne pathogens combined with the possibility of isolating intact microbes from part of the homogenized tick. The lysis buffer was essential during the bead-beating to preserve a high yield of RNA, which ruled out the possibility for TBEV isolation.
After bead-beating, the 48 tubes are centrifuged to pellet the tick remains, and the supernatant containing the genetic material is transferred by manual pipetting to new tubes. RNA is extracted from 48 samples at the same time using a commercial extraction kit using an extraction robot.

The aim has been to develop a method that is able to process many ticks in a short time as reproducible and efficient as possible, while reducing the risk of contaminating samples. To achieve this we aimed to automate as many steps as possible in the tick processing.

cDNA synthesis

To enable investigation of the genetic material from the ticks for many different pathogens at different time points we converted most of the RNA to complementary DNA (cDNA) which is more stable. All the pipetting steps to synthesize cDNA from 48 RNA samples at the same time were automated with a pipetting robot for precision and reproducibility.

Development of real-time PCR for detection and quantification of TBEV

In the beginning of my PhD-study I strived to develop a new real-time PCR assay for TBEV with the goal of being able to detect low copy numbers of TBEV in ticks with highest possible sensitivity. Primers were designed based on 74 sequences of TBEV-strains including all 3 subtypes present in the GenBank database at that time, which were scrutinized for suitable primer locations that would allow sensitive detection of all 3 TBEV subtypes. The only other real-time PCR assay published at that time was designed based on only 4 TBEV sequences (3 European and 1 Far-Eastern) [222]. The assay I designed was several times more sensitive than the other real-time PCR assay on several of the TBEV strains that I tested. However, the specificity was not sufficient due to my choice of using SYBR Green in the assay. The positive samples would have required sequencing for confirmation to distinguish between true and false positive results. I learnt from personal experience that all PCR assays have unspecific, unintended amplification, resulting from the primers attaching to other sites than the intended target. With SYBR Green, amplification of these unspecific PCR products also adds to the fluorescent signal. That is a problem when trying to detect very low gene copy numbers, with a low prevalence, as is the case with TBEV. The solution to this problem is the TaqMan assay, where there still is unspecific amplification that inevitably reduces the efficiency of the
amplification, like in all PCR assays, but with the difference that the chance of having a signal from an unspecific unintended amplification is infinitesimally small if the assay is correctly optimized. This is due to the requirement of the probe in the TaqMan assay that needs to attach between the 2 primers in order to produce a fluorescent signal. Essentially, with TaqMan, a signal is produced only from amplification of the target that the assay is designed to detect. SYBR Green type assays where the fluorescent molecules binds to and produces a signal from all double stranded DNA can be useful where absolute specificity is not paramount. Furthermore, SYBR Green is particularly useful as a step in the design of new Real-time PCR assays. It is then desirable to detect every amplification product that is formed in the reaction for the purpose of optimizing all parameters in the real-time PCR. In order to reduce unspecific amplification products as much as possible while keeping the reaction efficiency as high as possible several parameters needs to be optimized, i.e. primer sequence, primer GC content and length, annealing temperature, cycling conditions and optimal time for the different cycling steps.

The solution for detection and quantification of TBEV was to convert to the TaqMan real-time PCR assay. In 2010, a new TaqMan real-time PCR was published [126], and to make sure that we had the highest possible sensitivity for detecting different strains of TBEV, we evaluated both TaqMan assays on 12 TBEV strains that was serially diluted to determine the difference in lowest limit of detection for the 2 assays. The results showed that the assays differed significantly in detection limit for different TBEV strains (Table 4). By combining the primers from both assays into a multiplex assay, a cost and time-efficient method was obtained, without loss in sensitivity.


<table>
<thead>
<tr>
<th>Subtype</th>
<th>TBEV strain</th>
<th>The more sensitive assay</th>
<th>Higher dilution factor detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBEV-Eu</td>
<td>93-3511</td>
<td>Gäumann et al.</td>
<td>8 x</td>
</tr>
<tr>
<td>TBEV-Eu</td>
<td>Est 3051</td>
<td>Gäumann et al.</td>
<td>8 x</td>
</tr>
<tr>
<td>TBEV-Eu</td>
<td>Est 3476</td>
<td>Gäumann et al.</td>
<td>8 x</td>
</tr>
<tr>
<td>TBEV-Eu</td>
<td>Est 3509</td>
<td>Gäumann et al.</td>
<td>16 x</td>
</tr>
<tr>
<td>TBEV-Eu</td>
<td>Est 3053</td>
<td>Gäumann et al.</td>
<td>64 x</td>
</tr>
<tr>
<td>TBEV-Eu</td>
<td>9*000801</td>
<td>Schwaiger &amp; Cassinotti</td>
<td>8 x</td>
</tr>
<tr>
<td>TBEV-Eu</td>
<td>Lat 12849</td>
<td>Equal</td>
<td>Equal</td>
</tr>
<tr>
<td>TBEV-Eu</td>
<td>K 23</td>
<td>Equal</td>
<td>Equal</td>
</tr>
<tr>
<td>TBEV-Sib</td>
<td>Lat 1-96</td>
<td>Schwaiger &amp; Cassinotti</td>
<td>Only assay designed to detect</td>
</tr>
<tr>
<td>TBEV-Sib</td>
<td>Est 54</td>
<td>Schwaiger &amp; Cassinotti</td>
<td>Only assay designed to detect</td>
</tr>
<tr>
<td>TBEV-Sib</td>
<td>Est 3535</td>
<td>Schwaiger &amp; Cassinotti</td>
<td>Only assay designed to detect</td>
</tr>
<tr>
<td>TBEV-FE</td>
<td>Sofjin</td>
<td>Schwaiger &amp; Cassinotti</td>
<td>Only assay designed to detect</td>
</tr>
</tbody>
</table>
For quantification of TBEV gene copies in the real-time PCR and determining the reaction efficiency, a serial dilution of a plasmid standard with both target sequences of the multiplex TaqMan assay was used as a reference in each run. A benefit of having the target sequence of both primer pairs in the same plasmid is that it gives an exact comparison between the 2 primer pairs and the resulting fluorescent signal from amplification. A risk is that if there is a contamination by the plasmid in the lab space used for the real-time PCR setup, then the usability of both assays are compromised at the same time. It is of the utmost importance to have routines to prevent contamination when performing PCR assays. This is achieved by performing different laboratory steps in separate rooms, with different protective clothes in each room, changing gloves often, cleaning all lab spaces with chemicals and decontaminating with UV-light, and using only one-time plastics and filtered pipette tips free from RNase and DNase.

To set up the real-time PCR reactions a pipetting robot was used in order to eliminate manual liquid handling of the samples, which otherwise would increase random variation in the real-time PCR assay. The pipetting robot is used both in the cDNA synthesis and real-time PCR setup to ensure as little variation as possible when pipetting the different samples and reagents.

All samples were analyzed in duplicates and all positive findings were reanalyzed in triplicates to ensure correct quantification of the number of TBEV gene copies.

It is possible to use other methods than real-time PCR to address some of the questions we would like to answer. Nested PCR could be used for detection but not for quantification of TBEV in the ticks. Some studies have used ELISA to study the prevalence of TBEV in ticks [223]. ELISA is a semi quantitative method and the specificity contra sensitivity is often not as good as real-time PCR. It could also be possible to analyse the ticks by electron microscopy, this however would be very time consuming and expensive and could not be used for reliable quantification. It would also be possible to isolate TBEV from the ticks in mice or on cell culture, but this requires a bio safety level 3 laboratory because of the hazard classification when dealing with infectious TBEV.

The advantage of using PCR techniques is that it is fast, with great sensitivity and specificity, and that it is easy to sequence positive findings. This gives an extra confirmation, and it also enables further characterization of the strain we find through phylogenetic analysis.
Extraction and real-time PCR controls

For correct quantification, reproducibility and elimination of false positive and negative results, it is important to use internal controls (IC) and external controls (EC). One method would be to add an exact amount of an IC, ideally a virus with similar properties to TBEV, in an exact amount to each sample tube with lysis buffer, tick and steel bead. The idea is that this would be used to assess the combined efficiency of all steps in the tick processing, bead beating, RNA extraction and cDNA synthesis, and ensure reproducibility and correct quantification. This approach would give two possibilities in the real-time PCR analysis. Either to have a duplex real-time PCR where we have at least 2 primer pairs in the same reaction, both for detection and quantification of TBEV and for detection and quantification of the IC. This however, is not good in terms of reaction efficiency if there is a big difference in the amount of target TBEV versus target IC. With the real-time PCR described by Schwaiger and Cassinotti (2003) [222], it was possible to detect down to 10 copies of TBEV together with 50 copies of added IC, and up to 1000 copies of TBEV together with 50 copies of added IC. With a sample of more than 1000 copies of TBEV the IC was useless. This is because when one template in the PCR is dominating the exponential amplification, that template will hinder the DNA polymerase and substrates in the reaction to amplify the other templates. A possibility would be to check for TBEV and IC in separate reactions, however that would require twice as many reactions for each sample.

We decided to do a compromise and use both EC and IC. EC in the form of having 1 positive control, and 1 negative control in each extraction batch. Furthermore, an IC by running a separate real-time PCR on 2 randomly selected samples from each extraction batch targeted at a gene from the I. ricinus tick. In this way we make sure that there is no problem in each batch of 46 tick samples, without checking every sample individually. The reference plasmid standard used in every real-time PCR for quantification is also an EC of the real-time PCR, but not for the preceding RNA extraction and cDNA synthesis steps.

Cloning

The cloning technique was used in paper IV to determine the presence of quasispecies in single TBEV isolates (i.e. genomic differences among a population of virus copies). Amplified DNA from a PCR reaction is ligated into a plasmid cloning vector, where 1 plasmid only incorporates 1 PCR fragment. The plasmids are transfected to E. coli, where each bacterium only takes up 1 plasmid. The plasmid contains an antibiotic resistant gene, so bacteria that contain a plasmid can grow on
agar plates with antibiotic added, thereby selecting for those transfected. By selecting individual bacteria colonies and cultivating them further, the probability that each colony only contains 1 clone is high. When sequencing a large number of such clones, the sequence variation and proportion of different TBEV quasispecies can be determined.

**In vitro transcription**

In order to establish that the differences in sequence length observed over the poly(A) region in paper IV was not the result of polymerase stuttering, *in vitro* transcription was used to transcribe a DNA replicon of the TBEV strain Toro 2003 into RNA.

**Sequencing and phylogenetic analysis**

In papers II and IV, conventional sequencing was used to generate sequences directly from amplified PCR products. Software for multiple sequence alignment and phylogenetic analysis was used to characterize TBEV strains.

**Serological methods**

Antibodies against TBEV were analyzed in all serum samples in paper III using 3 different serological assays. Two of the assays were commercial ELISA assays for measuring anti-TBEV IgG titers that are widely used in clinical laboratories in Sweden. The third assay was a rapid fluorescent focus inhibition test (RFFIT) for detection of TBEV specific neutralizing antibodies, developed at the Swedish Institute for disease control. These 3 assays were used in order to evaluate TBEV antibody levels in serum using the most commonly used assays in Sweden and to compare them to each other. It is important to include an assay for measuring neutralizing antibodies since they are regarded as the best correlate of protection against TBEV infection.
Statistics

Statistical analyses were performed using GraphPad Prism for papers I and III, and using SPSS for paper III. P-values <0.05 were considered statistically significant.

In paper I, the chi-square test was used to assess association between categorical variables, i.e. feeding-time (categorized), tick life-stage, age group, tick attachment site, and gender. If a category contained less than 5 observations, the Fisher’s exact test was used instead of the chi-square test. The Spearman rank correlation test was used to determine if there was a correlation between age group and the proportion of nymphs and adult ticks removed >24h.

In paper III, the nonparametric Kruskal Wallis test was used to test differences in geometric mean antibody titer between groups. The Fisher’s exact test was used to test differences in serum positivity rate (SPR) between groups. Binary logistic regression was used to assess association between health-related factors and SPR. Multiple linear regression was used to assess association between health-related factors and antibody titer. Correlation between health-related factors was tested using the Pearson correlation test. The student t-test was used to test differences of mean age and mean number of vaccine doses between groups. The nonparametric Spearman rank correlation test was used to evaluate the correlation of titers measured using the different assays. A receiver-operating characteristic curve analysis was performed to evaluate sensitivity and specificity of the 2 ELISA assays in reference to the neutralization test.

The reason behind the large number of different statistical methods used in paper III was that 3 different serological assays were used, where 2 of the assays gave a ratio type dependent variable (titer), and all 3 assays gave a nominal type dependent variable, the determination of seropositivity, based on manufacturer specified cutoff levels. The multiple linear regression and correlation analysis methods was used due to the number of independent variables. Furthermore, additional statistical analysis was used for evaluating differences between the 3 serological assays.
Summary of results

Interactions between ticks and humans

The focus of paper I was to analyze interactions between ticks and humans based on the TBD STING-study material from 2008 – 2009, in the form of ticks collected and questionnaires filled in by tick-bitten participants. We analyzed 2110 ticks and filled in questionnaires from 1770 participants (648 men and 1122 women) in collaboration with 34 primary health care centers located in Southernmost Sweden, South Central Sweden, Northern Sweden, and the Åland Islands (Fig. 8). The median age of the participants was 63 years (range 19 – 92). Most of the ticks were nymphs (n = 1519), followed by adult females (n = 487), larvae (n = 89), and adult males (n = 15).

The seasonal distribution of tick bitten participants from the 4 geographical regions were investigated. In addition to the seasonal distribution of tick life stages, the distribution of attachment site on the human body for the 3 life stages of ticks, and feeding time with regard to feeding site, age and gender of the tick-bitten humans were also investigated.

All ticks collected were I. ricinus. The nymphal infestations were highest in June to July with a 2nd lower peek in August. The infestations from adult female ticks were highest from July to September. The tick season 2008 lasted between middle of May to middle of October, while the tick season 2009 spanned from early April to early November. The temperatures during April and November were higher during 2009 which could explain the longer tick season.

A higher proportion of adult female ticks were attached to the head/neck area, the upper body, and groin/genital area compared to nymphs (Fig. 10 A). Statistically higher proportion of ticks attached to the groin/genital area was found on men compared to women, and attached to the head/neck area on women compared to men (Fig. 10 B). The most frequent tick attachment site on both men and women were the legs, accounting for half of all investigated ticks (Fig. 10 B).
Among both nymphs and adult female ticks, 63% fed >24h before they were removed. Women removed a significantly higher proportion of nymphs before 24h of feeding (40%) than men (32%), and men removed both nymphs and adult ticks later than women on average (Fig. 11).

The time the nymphs fed before they were removed increased with age of the tick-bitten individual, both for men and women (Fig. 12). This could be due to poorer vision, more skin lesions that makes ticks harder to distinguish, and reduced physical sensitivity with increasing age. The duration of feeding might affect the risk of developing some TBDs [225]. It is therefore important to remove ticks as soon as possible.

**Figure 10.** (A) Percent of each tick life stage attached to different regions of the body. (B) Difference between tick attachments sites on men and women, based on all tick life-stages. (Figures 4 and 5 in paper I [224])
Figure 11. Mean feeding time with 95% CI of 1451 nymphs and 437 adult *I. ricinus* detached from men and women.

Figure 12. Linear regression of age against feeding time for 1451 nymphs and 437 adult *I. ricinus* detached from (A) men, and (B) women. The feeding time for nymphs increased significantly with increased age of the tick-bitten individual, both for men (17 min/year, p < 0.001), and women (10 min/year, p = 0.001).
TBEV in ticks detached from humans

The focus of paper II was to investigate the risk of human TBEV infection and development of symptoms after a bite by a TBEV infected tick using material collected in collaboration with 34 primary health care centers (Fig. 8) in the TBD STING-study 2008 and 2009. We wanted to investigate prevalence and quantity of TBEV in ticks that have bitten humans and determine if the tick feeding duration, tick life-stage and copy number of TBEV in the tick correlated to the risk of infection and development of clinical symptoms.

An important part of the study was to develop an optimized, efficient and reproducible method of extracting high quality RNA from the ticks, and a sensitive real-time PCR for detection and quantification of TBEV.

In total, 2167 ticks from 1886 tick-bitten humans were analyzed. Only 5 ticks were TBEV infected (Table 5). Three of them had bitten vaccinated individuals on the Åland Islands (Table 5 no. 2, 4, 5), one of which experienced clinical symptoms during the study period (Table 5 no. 2), but most likely from an ongoing *Borrelia* infection. The other 2 TBEV infected ticks had bitten unvaccinated individuals in Sweden. One of them got bitten by a tick containing 1800 virus copies with a feeding duration of 12-24h (Table 5 no. 3). Interestingly, this individual developed neither antibodies nor any clinical signs of infection. The other individual, a healthy 72 year old man, got bitten by a tick containing 7.7 million TBEV copies with a feeding duration of >60h (Table 5 no. 1). Unfortunately this man did not attend the 3 month follow up. Two years later contact with him was initiated, and blood samples and anamnesis could be collected. He told us that he had experienced fever and headache for a few days 1 week after the tick-bite. About 1 month after the tick-bite, he got problems focusing with his eyes and blurred vision. He visited a primary health care center were they took a blood sample for *Borrelia* serology, which was negative. He recovered and took 3 doses of TBEV vaccination the following year. The blood sample received 2 years after the tick-bite was strongly positive for TBEV IgG antibodies and negative for IgM antibodies, but if the seroconversion was due to a TBEV infection or the vaccination could not be established.
Two individuals with no detectable TBEV in their collected ticks got a TBEV IgG antibody seroconversion during the 3 month study period. An interview with these individuals confirmed that they had not been vaccinated during the study period. One of them reported no symptoms at all, while the other reported headache, fatigue, neck pain, vertigo, and concentration difficulties, but the symptoms started after several additional tick-bites during the study period. Six additional collected blood-fed nymphs were received and analyzed from this individual, all were TBEV-negative. Interestingly, both individuals that seroconverted had been tick-bitten in the same geographic region near Vinslöv, southern Sweden.

Interesting findings in this study were that virus copy number correlated with tick feeding-time (Table 5). Furthermore, one individual got bitten by a TBEV infected tick for 12-24h without getting infected, and antibodies against TBEV were produced during a completely asymptomatic infection. Being bitten by a tick containing several million TBEV copies only appears to have caused a 1\textsuperscript{st} phase viraemia, with perhaps mild CNS disturbance effecting the eyes, but not the 2\textsuperscript{nd} phase TBE (Table 5 no 1). However, no certain conclusions could be drawn since all of these results are based on few observations. Rather they can serve as hypothesis generating for future studies.

Table 5. Five ticks (all nymphs) positive for TBEV detached from humans. (Table 4 in paper II [220])

<table>
<thead>
<tr>
<th>No.</th>
<th>Region</th>
<th>Feeding-time (a) (h)</th>
<th>TBEV copies (b)</th>
<th>Tick co-infection (c)</th>
<th>Month of tick-bite</th>
<th>IgG seroconversion</th>
<th>TBEV vacc. before study</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C</td>
<td>&gt; 60</td>
<td>(7.7 \times 10^6)</td>
<td>Borrelia</td>
<td>July</td>
<td>ND(d)</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>D</td>
<td>24-36</td>
<td>(4.2 \times 10^3)</td>
<td>No</td>
<td>Aug.</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td>12-24</td>
<td>(1.8 \times 10^3)</td>
<td>No</td>
<td>June</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>D</td>
<td>&lt; 12</td>
<td>(&lt; 4 \times 10^2)</td>
<td>Borrelia</td>
<td>Aug.</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>D</td>
<td>&lt; 12</td>
<td>(&lt; 4 \times 10^2)</td>
<td>Anaplasma</td>
<td>Sept.</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

A: South Sweden, B: Middle Sweden, C: North Sweden, D: the Åland Islands, ND: not determined.
\(a\) Based on measurements of tick dimensions [221].
\(b\) TBEV copies in the tick calculated from a detection limit of 5 gene copies per PCR reaction.
\(c\) (Wilhelmsson et al. unpublished; Kozak et al. unpublished)
\(d\) The person did not attend follow-up.
Dose response of vaccination in older individuals

The focus of paper III was to investigate the dose response to TBEV vaccination in relation to 14 health-related factors, evaluated using 3 different antibody assays, for participants from the Åland Islands in the TBD STING-study 2008 – 2009. The Åland Islands are a highly TBE endemic region in the archipelago between Sweden and Finland. Therefore a mass vaccination program started in 2006 covering the population above 7 years old.

Blood samples, questionnaires and ticks were received from 533 individuals (353 women and 180 men) on the Åland Islands. The median age of the participants was 59 years for women and 64 for men. Approximately 80% of both genders were over 50 years old.

The 14 health-related factors were obtained through vaccination records and the TBD STING-study questionnaires, which included: age, gender, number of vaccine doses (0-5), time since last vaccine dose, previous TBE disease, vaccination against other flaviviruses, ≥2 tick-bites during the previous 3 months, pet-ownership, asthma, smoking, allergy, diabetes, medication, and previous tumor. The levels of TBEV IgG antibodies were measured in all serum samples using 2 commercial ELISA assays (Enzygnost and Immunozym), and TBEV neutralizing antibodies was measured using an in-house rapid fluorescent focus inhibition test.

![Figure 13](image_url)

**Figure 13.** Serum negativity rate in relation to number of vaccine doses (3 – 5), for participants ≥50 years of age.
The proportion of individuals 50 years or older that were seronegative for neutralizing antibodies less than 2.5 years on average from the last vaccination dose, with regard to number of vaccine doses received were: 47% (3 doses), 23% (4 doses), and 6% (5 doses) (Fig. 13).

**Figure 14.** Linear regression of age versus antibody titer. Grouped by study participants that have taken 3 (n = 227), 4 (n = 155) or 5 (n = 34) vaccine doses, analyzed using: (A) Enzygnost, and (B) Immunozym. (Figure 4 in paper III)
As expected, those who had received 4 or 5 vaccine doses had a significantly higher serum positivity rate. The response to vaccination declined linearly with age at the same rate on average for those who had received 3 vaccine doses compared to those who had received 4 vaccine doses (Fig. 14 A and B). There was no difference in antibody decline rate or overall antibody titer between women compared to men with regard to age, and no statistically significant decline of antibody titer with regards to time since the last vaccination (0 – 4 years) could be observed.

The 1st and 3 month serum sample from 3 TBEV vaccinated TBD STING-study participants that got bitten by TBEV infected ticks, as reported in paper II, were analyzed in parallel. Measured antibody titers were higher in the 1st serum samples taken a few days after the tick-bites compared to serum samples 3 month later. This can be explained by either a specific up regulation of TBEV antibodies from contact with the virus, or a more general up regulation of the immune system caused by the tick-bite, or be coincidental.

The 2 ELISA assays were compared to the neutralizing antibody test using receiver-operating characteristic curve analysis, to display tradeoff between sensitivity and specificity, and overall accuracy of the 2 tests. Overall accuracy of the 2 ELISA assays were the same, but the cutoff levels chosen by the manufacturers of the assays for determining if a test is positive or not differed considerably.

In conclusion, we found that the age of the individuals and the number of vaccine doses were the most important factors for successful vaccination. The response to immunization declined linearly with age. An older individual would on average need 4 vaccine doses to achieve the same antibody titer as somebody 35 years younger would after 3 vaccine doses. This suggests the need for an age adapted primary vaccination against TBEV.
Quasispecies of TBEV in blood-feeding ticks

The focus of paper IV was to investigate the presence of TBEV quasispecies from different sources, including ticks that have bitten humans in the TBD STING-study, questing unfed ticks and blood from humans with TBE.

During sequencing of the 3’ untranslated region (UTR) of the TBEV strain Saringe-2009 from a tick that had been feeding on a 72 year old man >60h that participated in the TBD STING-study (reported in paper II), the height of the fluorescent peaks in the sequencing reactions in both directions gradually declined and disappeared over the poly(A) region. This indicated that there were DNA fragments of different lengths over this region. To resolve the sequence and determine the distribution of sequences with different poly(A) length, PCR products from this region were inserted into plasmid cloning vectors, allowing single clones of the plasmids containing 1 PCR fragment each to be transfected to E. coli, amplified and sequenced.

This revealed that the Saringe-2009 TBEV strain contained a heterogeneous population of quasispecies, with varying length of the poly(A) region, between 10 to 36 nucleotides (nt) long.

To find a plausible explanation for this quasispecies variance in the blood-feeding tick, the same cloning procedure and sequencing was performed on an additional 7 TBEV strains isolated from different sources (Fig. 15). Approximately 30 clones were sequenced from each strain.

The results of the comparison indicated that there exists a considerable variation in the variable 3’ UTR. This was demonstrated in blood-feeding ticks (Fig. 15 A, B), questing unfed ticks (Fig 15. C, D, E), and in TBEV strains isolated from human serum using suckling baby mice, followed by propagation in cell culture (Fig 15. F, G, H).

A continues range of quasispecies was only observed in the tick that had been feeding for >60h. The hypothesis is that this TBEV strain was caught in a moment when the pool of TBEV quasispecies rearranges from virus adapted to replicate in tick cells, to virus adapted to replicating in mammalian cells.

Previous observations have revealed that a tick adapted TBEV strain needs to propagate in mice a number of times to become pathogenic to mice, and that TBEV passaging in cell culture and/or mouse brain can result in spontaneous genomic deletions and elongations within the variable 3’UTR [56,57]. In addition, the variable 3’UTR has been shown to be a critical virulence factor for the Far-Eastern TBEV subtype [226]. However, this kind of heterogeneous population of quasispecies, that only differs in the poly(A) region, has not been observed previously.
By clarifying that the TBEV strain Habo 2011 (Fig. 15 B), with a short (6 nt) poly(A) tract, from a tick that had been feeding for 12-24h, caused TBE in the human bitten by that tick (unpublished data). One common denominator can be found among the characterized TBEV strains. All of those viruses that caused TBE in humans have a short (6 nt) poly(A) tract (Fig. 15 B, C, F, G), or a truncated variable 3'UTR where the poly(A) tract is completely missing (Fig. 15 H).

However, TBEV strains with a short or missing poly(A) tract was also isolated and sequenced directly from questing ticks (Fig. 15 C, D, E). This suggests that if a short or missing poly(A) tract has a connection to pathogenesis in humans, there are also questing ticks that harbors predominantly those TBEV strains, and a rearrangement of quasispecies would not need to take place before becoming pathogenic to humans. It is possible that TBEV strains with a longer poly(A) tract circulates in nature, that may be less pathogenic to humans, or requires a quasispecies rearrangement in order to become pathogenic. This could be the case with the Saringe 2009 strain (Fig 15 A), that had a heterogeneous population of quasispecies with variable poly(A) tract, isolated from a tick that had been feeding for >60h on a individual that only got the 1st viraemic phase with headache and fever, but did not develop TBE. If the length of the poly(A) tract in the variable region of the 3'UTR is connected to pathogenesis in humans or not needs to be further studied.
Figure 15. Schematic representation of the 3'UTR of The 8 TBEV strains (A – H) with isolation source and passage history mentioned under the strain name, in reference to the GenBank sequence Neudoerfl U27495. (A) had a variable poly(A) tract, and (E) consisted of 2 genetic variants, based on sequencing of 23 and 7 clones respectively. Strains (E – H) have been passaged in suckling baby mice and in cell culture. The passage history for (E) and (H) is unknown. The sequence elements depicted are repetitive sequences (R1, R2 and R3), the poly(A) tract, and cyclization motif (CSb-1).
Concluding remarks

TBE is a serious neurological disease that afflicts 200 – 300 individuals in Sweden each year, and approximately 10,000 individuals per year on the Eurasian continent. Therefore, it is important to study how ticks interact with humans in order to assess the risks of developing TBE in populations living in endemic areas. The results in paper I showed that the time until the tick was removed increased with increasing age of the human host. Furthermore, men on average removed their ticks later than women. These results could have serious implications since the risk of TBEV infection might increase with increasing tick feeding-time, and the virus may be able to replicate more in the tick before infecting the human host. In paper II, observations supporting both of these hypothesis were made. Although only 5 ticks that carried TBEV could be identified, a correlation was observed between tick feeding-time and copy number of TBEV in the ticks. Another observation was that one individual in the study had been bitten by a tick with a low copy number of TBEV (1800 copies) with a feeding time of 12-24h, and it appears that the virus weren’t transmitted to the tick-bitten individual. This contradicts previous results from an experimental study, where TBEV-infected ticks feeding on mice, transmitted the virus within minutes of the initiation of feeding [65]. However, the way the ticks were infected with TBEV in that study was artificial, and the virus load was high. It might be possible that naturally TBEV infected ticks do not transmit the virus directly. These observations highlights the importance of removing ticks as soon as possible, not just to lower the risk of Borrelia infection [225], but possibly also TBEV infection. Removing a blood-feeding tick quickly would also be important if the virus replicates during feeding, as a higher virus copy number likely increases the risk and severity of infection.

Older individuals have a higher risk of developing TBE [179], and the severity of the disease increases with age [175,177,178]. Furthermore, almost twice as many men as women develop TBE [180,181]. One reason for this could be the observation in paper I, that older individuals in general, and males in particular, discover and removes their ticks later.

Another factor that could be connected to why older individuals are more prone to develop TBE, and the severe forms of TBE, is the deterioration of the immune system with increasing age, which makes older individuals more susceptible to infections and to developing diseases [227–229]. This is also an important factor to consider in preventing infectious diseases through vaccination. In paper III, we observed that the immune response to TBEV vaccination declines linearly with age, and that older individuals, on average, needs to take 4 vaccine doses to acquire equal antibody levels as a 35 years younger individual after only 3 vaccine doses. A primary
vaccination against TBEV consists of 3 vaccine doses, regardless of the age of the individual being vaccinated. Stockholm County (Sweden) is an exception where 4 doses are recommended in the primary vaccination for individuals over 60 years old. Accounting for the observations in paper III, that 47% of the individuals over 50 years old were seronegative, despite having taken their latest vaccination dose on average 2.5 years before entering the TBD STING-study, an age adapted primary immunization should be considered.

The TBEV strain from a tick that had bitten a TBD STING-study participant (reported in paper II) was genetically characterized in paper IV. The tick had a long feeding time (>60h) before it was removed and the investigation revealed that the tick contained a heterogeneous population of TBEV quasispecies with variations in the poly(A) tract of the 3' UTR of the viral genome. This point to the possibility that quasispecies rearrangement takes place in the tick during feeding, where the virus adapts from replication in tick cells to replication in mammalian cells, which may have importance for the pathogenesis of the disease. This could again highlight the importance of removing an infected tick as soon as possible, before such rearrangement have time to take place, possibly making the TBEV more virulent. In paper IV, 7 other TBEV strains was genetically characterized for comparison. Similarities in the 3'UTR between the TBEV strain Mandal 2009, isolated from questing ticks in Norway, and the highly virulent TBEV strain Hypr, that was isolated from a diseased TBE patient in 1953, suggested that highly virulent TBEV strains may already circulate in nature and be present in questing ticks.

Of the 8 characterized TBEV strains in paper IV, 5 strains had infected humans. Four of these had a short, 6 nt long poly (A), or completely lacked the poly(A)region, and these strains caused TBE in the human. One TBEV strain did not cause TBE in the infected human. This strain had a longer heterogeneous poly(A) tract (10 – 36 nt). This may be a coincidental observation or it might point to the poly(A) region being implicated in the pathogenesis of TBEV in humans. The 3'UTR has been shown to be a critical virulence factor in TBEV of the Far-Eastern subtype in studies om mice [226]. Further characterizations of the 3'UTR in TBEV strains from both symptomatic and asymptomatic humans needs to be conducted in order to clarify if the poly(A) region affects TBEV virulence.
Future perspectives

Concerning TBEV infections in humans, we still know very little about what factors decides who develops TBE and who only develops a mild phase I illness, or an asymptomatic infection.

We need to study and characterize the dynamics of TBEV replication in ticks that are feeding to understand how the feeding-time affects the viral load in the ticks, and also to characterize quasispecies changes in TBEV during the feeding. Then we can assess how this contributes to the infection dynamics and virulence.

To do this I think we need to set up experimental models. This could be done with an artificial tick feeding system, using a source of blood separated by a membrane, through which the ticks can feed in a controlled environment. Furthermore, the replication dynamics of different TBEV strains could be studied in cell cultures of different types of tick and mammalian cell lines.

Many more TBEV strains needs to be sequenced, directly from questing unfed ticks, from ticks after different durations of blood feeding, and TBEV strains propagated in tick cell cultures versus mammalian cell cultures. This could be used to elucidate mechanisms behind the differences observed in the variable 3’ UTR and the role of quasispecies in the TBEV replication cycle between different host cells, and how this affects the replication dynamics and virulence.

Another field to study should be host genetic factors. For predicting the risk of developing TBE, and severe forms of TBE, blood samples from individuals that have developed TBE could be analyzed. Host genetic factors could also be studied to find explanations for individual differences in immunogenic response to TBEV vaccination.
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