University of
South-Eastern Norway

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Tick-borne encephalitis virus: an emerging virus in Scandinavia

Dissertation for the degree of Ph.D
Ecology

Faculty of Technology, Natural Sciences and Maritime Sciences

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Faculty of Technology, Natural Sciences and Maritime Sciences
University of South-Eastern Norway
Bø

Doctoral dissertations at the University of South-Eastern Norway no. 190

ISSN: 2535-5244 (print)
ISSN: 2535-5252 (online)

ISBN: 978-82-7206-840-9 (print)
ISBN: 978-82-7206-841-6 (online)

Print: University of South-Eastern Norway

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Dedication

To all those helping hands that made this academic journey possible.

Acknowledgements

I would like to express my gratitude to the University of Southeastern Norway (USN) and the Norwegian Institute of Public Health (NIPH) for providing me with the opportunity to pursue my PhD. This PhD study is one of the several larger projects funded by the Norwegian Ministry of Health and Care Services, Barents region program (grant number B1412 and B1710), EU Interreg V-A program Scandtick Innovation project (grant number 20200422); TBFVnet (2018-1-0659 EEA Norwegian grant for regional cooperation) and Swedish research council FORMAS (grant number 2015-710).

I want to thank my supervisors. Åshild K. Andreassen, my main supervisor, has been kind, consistently accessible, offering valuable insights, effective problem-solving, and genuine enthusiasm for my research. I extend my gratitude to my co-supervisors: Rose Vikse, Andrew Jenkins, Arnulf Soleng, Kristian Alfsnes, John Pettersson and Hans Renssen for their contributions to the research, manuscript revisions, and support. I would like to thank all the co-authors for valuable cooperation and contribution to this study.

I would like to thank my family and friends for their invaluable support. I am grateful for encouragements from my mother, siblings, and family in-law. I would like to thank my husband and my daughter, "Radha," for having unwavering faith in me and for generously allowing me to dedicate weekends to work. You are my champions, and I owe this success to you.

I am thankful to the tick collection team, particularly Arnulf Soleng and Kristin Edgar. I appreciate the company and lively moments with fellow mates including Katrine Mørk Paulsen, Benedikte N. Pedersen, Srijana Bastakoti, Eileen Stanzen, Malene Dieseth, Kamilla Hauser, Maria Diekmann and Urusha Maharjan. Special gratitude to Benedikte N. Pedersen for your warm hospitality and sharing apartment during my stay at Bø.

I want to acknowledge the Erasmus plus higher education mobility program at USN for enabling my visit to Umeå University, Sweden. A special thanks to Anna Överby Wernsted at for granting me access to her lab, to Richard Lindquist for his patient guidance in the TBEV neutralization test and Olivia Wesula Lwande for lovely evening out in Umeå Center. I wish to express my gratitude to the National Doctoral Programme in Infection and Antibiotics for providing me opportunities to both present my research and participate in academic events.

Lastly, I want to convey my gratitude to everyone at the Virology and Pest Control departments of NIPH for fostering a welcoming, friendly, and supportive environment throughout my PhD study.

01.12.2023, Oslo

Alaka Lamsal

Abstract

Tick-borne encephalitis virus (TBEV) causes Tick-borne encephalitis (TBE) which is an infection of the central nervous system that can lead to encephalitis. TBE cases are increasing in Scandinavia over the last few decades posing a concern to public health. The geographical spread of TBEV can be caused by favourable climatic conditions due to ongoing climatic changes. This PhD thesis aims to gain a comprehensive understanding on the status of TBEV distribution in Norway, Sweden, and Denmark.

TBEV distribution differs along geographical regions in Scandinavia due to distinct climatic and ecological conditions. TBEV circulation was found to be widespread in coastal and lowland areas, with multiple foci capable of initiating endemic regions. In alpine regions, where little is known about tick distribution, reindeer were sero-screened as sentinel animals. Antibodies against TBEV was not detected in reindeer populations indicating that tick that carry TBEV may not have reached in reindeer foraging regions. However, the inclusion of recent data and other deer species as sentinels for monitoring could be helpful.

Human incidence of TBE clusters around specific coastal regions despite widespread geographical distribution of TBEV. The TBEV-Eu strain often presents with mild symptoms, that can be mistaken as flu or other illness. Retrospective sero-analysis of patients with CNS symptoms found one underreported case that show loopholes in TBE reporting system. This resonates with underestimation of TBE cases concluded from earlier studies.

A whole genome sequencing methodology for TBEV strains was established in the reference laboratory for TBE in Norway. This approach facilitates bioinformatic and phylogenetic analysis which are important for tracking TBEV spread and its future trajectory in Scandinavia. This established technique can serve as a reference for sequencing low viral load strains typical from ticks and human cases.

Keywords: *tick-borne encephalitis virus, zoonoses, climate, whole genome sequencing, sentinel.*

List of papers

Paper I

Lamsal, A., Edgar, K. S., Jenkins, A., Renssen, H., Kjær, L. J., Alfsnes, K., Bastakoti, S., Dieseth, M., Klitgaard, K., Lindstedt, H. E. H., Paulsen, K. M., Vikse, R., Korslund, L., Kjelland, V., Stuen, S., Kjellander, P., Christensson, M., Teräväinen, M., Jensen, L. M., Bødker, R., Soleng, A., Andreassen, Å. K. (2023). Prevalence of tick-borne encephalitis virus in questing *Ixodes ricinus* nymphs in southern Scandinavia and the possible influence of meteorological factors. *Zoonoses and Public Health*, 70, 473–484. https://doi.org/10.1111/zph.13049

Paper II

Lamsal, A., Tryland, M., Paulsen, K. M., Romano, J. S., Nymo, I. H., Stiasny, K., Soleng, A., Vikse, R., & Andreassen, Å. K. (2023). Serological screening for tick-borne encephalitis virus in eight Norwegian herds of semi-domesticated reindeer (*Rangifer tarandus tarandus*). *Zoonoses and Public Health*, 70, 692–698. https://doi.org/10.1111/zph.13060

Paper III

Lamsal, A., Diekmann, MJ., Paulsen, E.Q., Dorenberg, D.C.H., Růžek, D., Salat, J., Vikse, R., Soleng, A., & Andreassen, Å. K. A retrospective study on the prevalence of TBEV-specific antibodies in patients with CNS symptoms admitted to Oslo University Hospital, Oslo Norway. *Manuscript draft*.

Paper IV

Paulsen, KM., Lamsal, A., Bastakoti, S., Pettersson, J. H., Pedersen, B.N., Stiasny, K., Haglund, M., Smura, T., Vapalahti, O., Vikse, R., Alfsnes, K., & Andreassen, Å. K. High-throughput sequencing of two European strains of tick-borne encephalitis virus (TBEV), Hochosterwitz and 1993/783. Ticks and Tick Borne Diseases, 2021 Jan;12(1):101557. doi: 10.1016/j.ttbdis.2020.101557.

Summary of papers

Paper I

Paper I investigated the geographical distribution of TBEV in low-lying coastal terrains of southern Scandinavia, conducive for tick habitats. Questing nymphs were collected from 11 locations from southern Norway, 9 from southern Sweden and 30 locations from Denmark during autumn 2016. The samples were screened for TBEV using RT-real-time PCR and pyrosequencing for confirmation.

TBEV was detected in 20 out of 50 sites tested, indicating widespread distribution of TBEV in southern Scandinavia. An overall TBEV prevalence was 0.1%, with certain regions having prevalence above 0.5% such as along the Oslo fjord in Norway and uppermost parts of Skåne county in Sweden. TBEV prevalence was detected along several locations in Denmark. The TBEV prevalence did not show spatial patterns suggesting factors other than tick distribution, like climate may affect tick distribution. TBEV prevalence correlated with relative humidity suggesting humidity, may influence TBEV prevalence within tick populations.

Paper II

Paper II was conducted to understand the status of TBEV in alpine regions of Scandinavia. A total of 480 reindeer sera collected from eight herds during 2013-2015 were sero-screened for antibodies against TBEV. None of the reindeer were positive for TBEV specific antibodies. This indicates that TBEV may not have reached alpine regions. We found 16 TBEV-reactive samples which were negative in the serum neutralization test (SNT). This suggests a different flavivirus, closely related to TBEV may be circulating in the area.

Paper III

Paper III was performed to investigate the prevalence of TBE infections in patients with central nervous system (CNS) symptoms admitted to hospital. A retrospective sero-screening was carried out with ELISA IgG test followed by SNT and IFA assay. Out of 137 samples, one was TBE confirmed. On going back to the history, the TBE case tested positive in the laboratory but was failed to be reported to MSIS. Although this aligns with studies reporting possible underestimation of TBE cases, the result may imply only a minor underestimation of TBEV infection.

Paper IV

The primary objective of Paper IV was to develop a WGS method for TBEV at the Norwegian Institute of Public Health, the reference laboratory for TBEV in Norway. Two cultured TBEV strains were sequenced with primers producing overlapping fragments (Table 1, Paper III). Hochosterwitz strain from Austria was isolated from a tick and the 1993/783 was isolated from a patient in Sweden. Complete polyprotein sequences were obtained from both the strains. Subsequent phylogenetic analysis showed that both strains belong to the TBEV-Eu subtype where Hochosterwitz closely aligned with two strains from South Korea, while 1993/783 aligned with strains from the Netherlands. Ambiguous nucleotides were observed in raw sequence data in both the strains. Hochosterwitz contained 52 ambiguous nucleotides and 1993/783 had eight. These ambiguities could arise from PCR errors, sequencing mistakes, the virus passage history, or genetic variants.

List of abbreviations and definitions

bp	Base pair
cDNA	Complementary deoxyribonucleic acid made by reverse transcription of RNA
CNS	Central nervous system
Co-feeding transmission	When an infected tick and uninfected tick(s) are feeding in close proximity, or in quick succession, the transmission of TBEV through co-feeding may occur independently of host viremia.
Counties	There are 11 counties in Norway (Troms and Finnmark, Nordland, Trøndelag, Møre og Romsdal, Vestland, Rogaland, Agder, Vestfold og Telemark, Viken, Oslo, Innlandet), 21 counties in Sweden (Skåne, Hallands, Kronobergs, Kalmar, Blekinge, Jönköpings, Gotlands, Östergötlands, Örebro, Södermanlands, Uppsala, Västmanlands, Västra Götaland, Stockholms, Värmlands, Dalarnas, Gävleborgs, Västernorrlands, Jämtlands,Västerbottens, Norrbottens) and 5 administrative regions in Denmark (Hovedstaden, Sjælland, Syddanmark, Midtjylland, Nordjylland).
Ct	Cycle threshold
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
ECDC	European Centre for Disease Prevention and Control
ELISA	Enzyme-linked immunosorbent assay

Endemic region	According to the ECDC, a region is considered TBE endemic if human TBE cases occur on a regular basis.
Enzootic region	A TBE enzootic region is an area where TBEV infection is consistently present in tick and animal populations.
Foci	TBEV exists in foci, which are confined regions where the virus circulates among tick vectors and reservoir hosts.
FFA	Focus forming assay
GDD	Growing degree days represent a measure of accumulated heat units over time, typically used in agriculture to estimate plant growth and development based on temperature data.
GSL	The length of growing season is the time period between the last and first frost dates when the climate is suitable for plant growth.
HI	Haemagglutination inhibition test
Host	Tick hosts include all species that tick feed in nature.
IFA	Immuno-florescence assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
INDELs	Insertions/deletions
LIV	Louping -ill virus
masl	Meters above sea level
MIR	Minimum infection rate

MSIS	Meldingssystem for smittsomme sykdommer (Norwegian Surveillance System for Communicable diseases)
NCR	Non-coding region
NDVI	Normalized difference in vegetation index
NGS	Next-generation sequencing
PCR	Polymerase chain reaction
PRNT	Plaque reduction neutralization test
RCP	Representative concentration pathways are climate models predicting future greenhouse gas levels and climate impacts. “RCP8.5” predicts high emissions without human intervention, “RCP6” assumes stabilization with technology by 2100, “RCP4.5” assumes emission reductions after 2040, and “RCP2.6” represents drastic cuts in emissions starting in 2020.
qPCR	Quantitative polymerase chain reaction is a technique for amplifying and quantifying DNA often referred to as real-time PCR where amplification can be monitored in real time.
Reservoir host	A reservoir host is an organism that carries a pathogen without getting sick and can pass it to other species, often through vectors like tick and mosquitoes.
RH	Relative humidity
RNase	Ribonuclease
RSSE	Russian-Spring Summer Encephalitis
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Saturation deficit

SNT	Serum neutralisation test
SNVs	single nucleotide variants (SNVs)
T	Temperature
TBEV-Eu	Tick-borne encephalitis virus, European subtype
TBEV-Fe	Tick-borne encephalitis virus, Far-eastern subtype
TBEV-Sib	Tick-borne encephalitis virus, Siberian subtype
Treeline	Treeline is the edge of the habitat where trees are capable of growing
Vector	Vectors transmit pathogen from one host to another. Ticks qualify as a vector as it: (i) can consume blood from infected hosts; (ii) can acquire pathogen during the blood-feeding; (iii) can retain pathogen across one or more life stages (transstadial passage); and (iv) can transmit it to other hosts during subsequent feedings.
RNA	Ribonucleic acid
UTR	Untranslated region
WGS	Whole genome sequencing
ØKS	Øresund, Kattegat, and Skagerrak; contiguous sea areas between Norway, Sweden and Denmark

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1. Introduction

Tick-borne encephalitis (TBE) is a tick-borne zoonosis and a public health concern in Europe (Riccardi et al., 2019; Slunge et al., 2022). The causative agent is tick-borne encephalitis virus (TBEV) which can lead to a spectrum of clinical outcomes from mild flu like symptoms to severe neurological complications (Gritsun et al., 2003; Kaiser, 2002).

Scandinavia is the northern limit of habitat tolerance range for tick due to its cooler climate and distinct ecosystems (Jaenson & Wilhelmsson, 2019; Soleng et al., 2018). TBE is endemic in the coastal regions of southern Norway and the south-central parts of Sweden (MSIS, 2023; Public Health Agency of Sweden, 2023b). In recent decades, there has been a notable increase in TBE cases in Scandinavia indicating a shift in the epidemiological landscape of TBE (MSIS, 2023; Public Health Agency of Sweden, 2023a; Statens Serum Institut, 2023). This could be due to increased recreational activity in tick abundant areas or improved diagnostics and reporting of TBE (Slunge et al., 2022). It could also be due to changes in climatic conditions due to the global climate change (Tokarevich et al., 2011;Van Oort et al., 2020). As temperatures rise and precipitation patterns alter, tick habitats might be expanding, potentially ushering in a new epidemiological landscape for TBE (Alkishe et al., 2017; Lindgren et al., 2012). TBE may have the potential to spread to new areas or populations, and this spread might not be solely due to changes in the environment but could also be due to the virus evolution and host adaptation (Helmová et al., 2020; Liebig et al., 2020, 2021).

TBEV is enzootic along the coastline of Norway and Sweden (Pettersson et al., 2014; Soleng et al., 2018; Vikse et al., 2020). Antibodies against TBEV have been detected in both animals and healthy blood donors, suggesting broader exposure than the restricted known endemic areas (Andreassen et al., 2012; Larsen et al., 2014; Marvik et al., 2021; Paulsen et al., 2020). The future climate predictions project conditions that are both warmer and wetter in Scandinavia (Box et al., 2019; Christensen et al., 2022; Easterling et al., 2000; Hanssen-Bauer et al., 2017). Such climatic shifts could offer conducive environment for tick proliferation (Cayol et al., 2017; J. S. Gray et al., 2009; E. Korenberg, 2009; Lindgren et al., 2012; Van Oort et al., 2020; Zeman & Beneš, 2004). The warmer conditions implies a potential increase in abundance and geographical distribution of tick and TBEV throughout Scandinavia, subsequently, escalating the risk of human TBEV-infections in future (Daniel et al., 2018; Heyman et al., 2010; Lindgren et al., 2012).

1.1 Tick-borne encephalitis virus

The medical history of TBEV started from 1937 when it was first discovered by Professor LA Zilber in the Russian far-east (Kahl et al., 2023). Earlier, TBE was known as Russian spring-summer encephalitis in Russia and far-eastern Asia, and as central European encephalitis in Europe (Calisher et al., 1989; Ruzek et al., 2019). It is transmitted to humans mostly via infected tick bites but can also infect humans via consumption of unpasteurized dairy (Klaus et al., 2012; Omazic, et al., 2023; Paulsen et al., 2019).

Genomic structure

TBEV is a (+) sense, single-stranded RNA virus with an approximate genome size of 11 kb. The TBEV genome encodes a single polyprotein which is processed into three structural proteins (C, M and E) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) by cellular and viral proteases (Figure 1) (Barrows et al., 2018; Kutschera & Wolfinger, 2022). The genome of TBEV-EU has conserved 5' and 3' untranslated regions (UTR), which have roles in replication, genome packaging, affect immune response, virulence and pathogenicity (Barrows et al., 2018; Kutschera & Wolfinger, 2022; Pulkkinen et al., 2018).

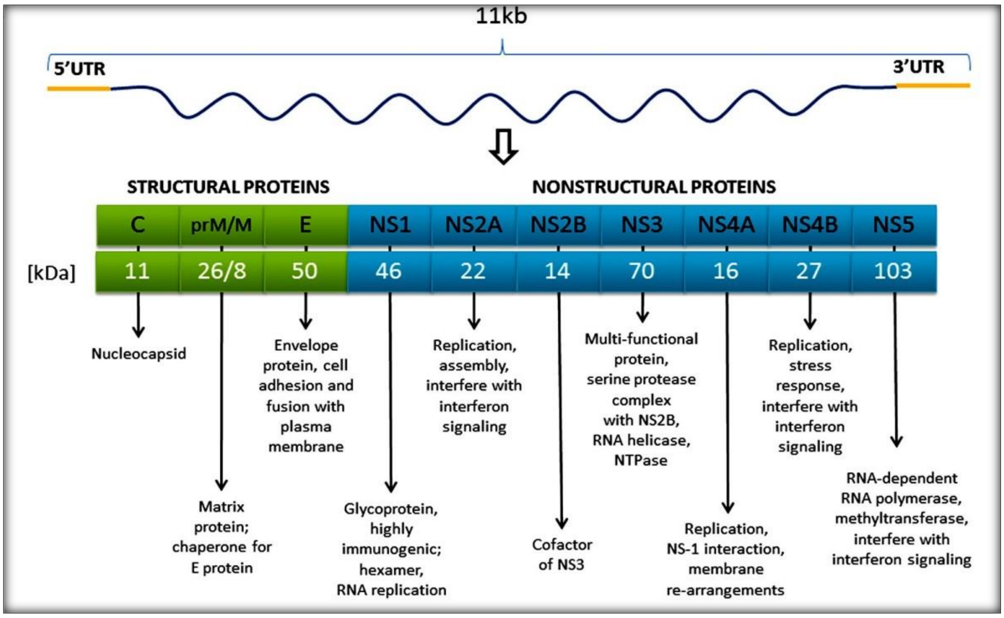

Figure 1: TBEV genome encodes a single polyprotein that is subsequently cleaved into three structural proteins and seven non-structural proteins. The 5' and 3' untranslated regions (UTRs) flank the coding region and play vital roles in viral replication and translation. The structural proteins facilitate viral entry and assembly while the non-structural proteins are primarily involved in replication and evasion of host immune responses. *(Kindly provided by Martin Palus, with permission).*

The envelope protein plays an important role in infection allowing virus binding to recept ors on the surface of susceptible host cells (Barrows et al., 2018). The non-structural protein (NS) play a role in the viral pathogenicity and can be a determinant in triggering immune responses (Pulkkinen et al., 2018; Starodubova et al., 2023). RNA viruses including TBEV exhibit high mutation rates, attributed to the absence of proofreading mechanisms during virus replication (Domingo, 1997; Helmová et al., 2020).TBEV can develop distinct viral variants demonstrating its genomic flexibility and ability to form quasispecies within a virus population (Asghar et al., 2014; Helmová et al., 2020; Schneider & Roossinck, 2001).

Subtypes

TBEV is classified into several subtypes: European (TBEV-Eu), Siberian (TBEV-Sib), Far Eastern (TBEV-Fe), Baikalian (TBEV-Bkl) and Himalayan TBEV (TBEV-Him) (Ecker et al., 1999). The TBEV-Sib, TBEV-Fe and TBEV-Bkl subtypes are primarily transmitted by *I. persulcatus* (Demina et al., 2010; Kovalev & Mukhacheva, 2017; Kozlova et al., 2013). The Himalayan TBEV (TBEV-Him) is hosted by the Himalayan marmot (*Marmota himalayane*) which is found in the western part of China (Dai et al., 2018). TBEV-Eu can be vectored by *I. ricinus* and *I. persulcatus* and hybrid between these two (Belova et al., 2023; Kovalev et al., 2016).

Clinical manifestations

The clinical manifestations of TBE vary by subtype. The European subtype typically induces a milder form of the disease, and with a mortality below 2% (Gritsun et al., 2003; Kaiser, 2002; Ruzek et al., 2019). The Far Eastern and Siberian subtypes lead to a more severe form of illness (Ruzek et al., 2019). TBE can present asymptomatically; as a brief febrile illness; or as a central nervous system infection, often following a two-phase pattern (Kaiser, 2002; Lindquist & Vapalahti, 2008). The median incubation period is eight days, with the initial phase include fever and flu-like symptoms followed by a symptom-free period of one week. Then symptoms recur, indicative of central nervous system inflammation, such meningitis, meningoencephalitis, or, more rarely, encephalomyelitis (Lindquist & Vapalahti, 2008). Even though initial symptoms like fever, headache, muscle pain, and fatigue are common in viral infections, in severe cases the European subtype, can lead to encephalitis causing confusion, seizure, paralysis and potentially death (Gritsun et al., 2003). Clinical TBE cases are common between April and November peaking in autumn (ECDC, 2021; MSIS, 2023).

There are no specific treatments for TBE. Supportive care to manage symptoms remains the primary approach. TBEV vaccines have been developed and are recommended for those residing in or travelling to areas where the virus is endemic (Amicizia et al., 2013; Kunze et al., 2022). Despite the vaccine's effectiveness, challenges in achieving extensive coverage, particularly in high-risk areas and groups, persist (Kunze et al., 2022).

1.2 The tick vector

Tick serves as vector for transmission of TBEV. Infected tick carry TBEV in their midgut and salivary glands and transmit it to hosts during bites (Benda, 1958; Lejal et al., 2019). There are over 700 species of hard tick (Ixodidae) and 190 species of soft tick (Argasidae) (Madder et al., 2014). Tick species such as *Ixodes ricinus, Ixodes persulcatus, Dermacentor reticulatus, Ixodes trianguliceps, Ixodes hexagonous* are detected in Scandinavia. *I. ricinus* is the main carrier of TBEV in this region (Asghar et al., 2014, 2014; Jaenson & Wilhelmsson, 2019). Some of the details of the life cycle, particularly with reference to environmentally determined developmental diapause apply only to *I. ricinus,* which is more developmentally flexible than *I. persulcatus* (Korenberg et al., 2021). Thus, *I. ricinus* is preferable to 'tick' in the subsequent sections.

Life cycle

Tick undergoes a life cycle comprising egg, larvae, nymph, and adult stages (Figure 2). Excluding egg, each stage requires a blood meal to transition to the next stage. After feeding, tick find refuge in leaf litter for protection against sunlight and dehydration. They favour deciduous forested areas particularly those with oak (*Quercus* spp.) and beech trees (*Fagus sylvatica*). Deciduous trees serve as optimal habitats due to shade and humidity in Europe (Tack et al., 2012; Zając et al., 2021).

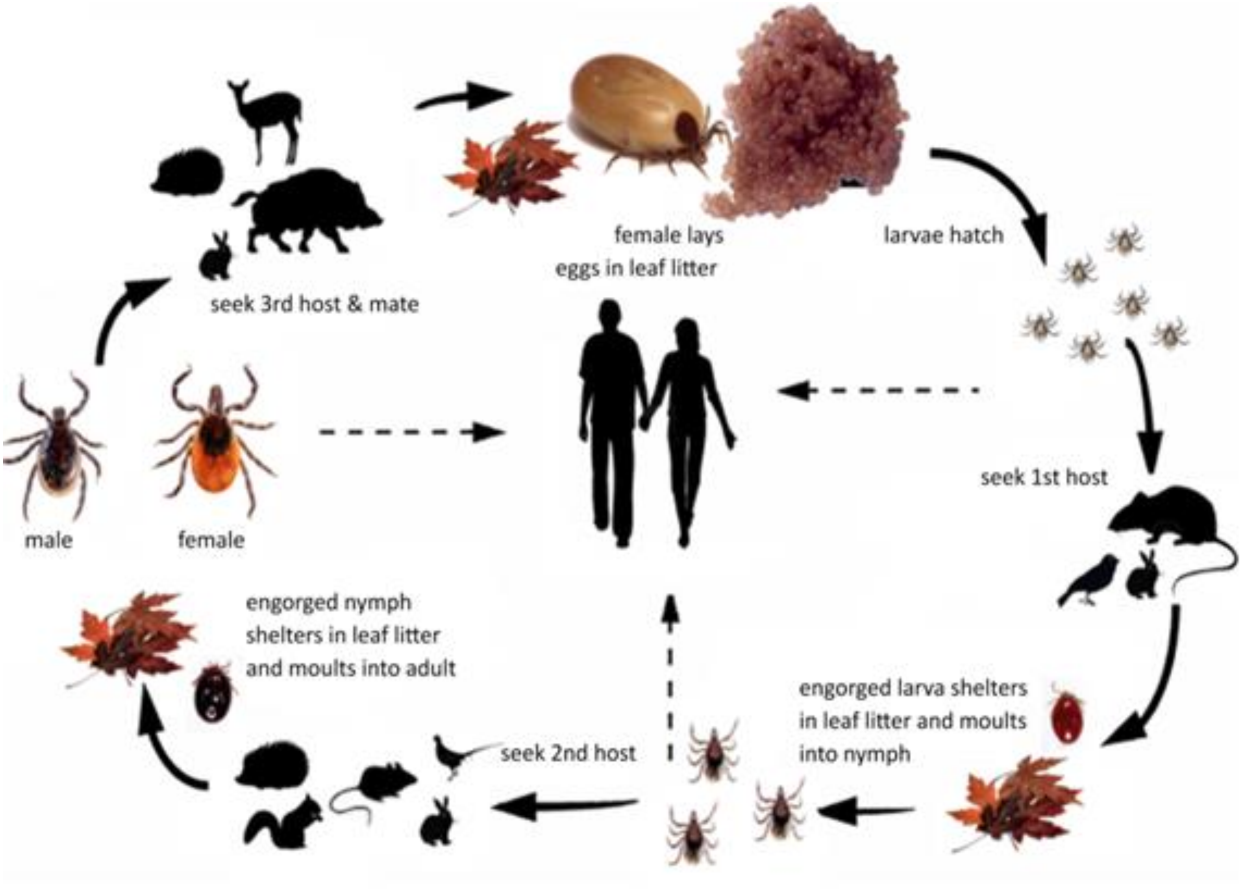

Figure 2: Life cycle of tick. Egg hatches into six-legged larva which after feeding molts to the eight-legged nymph. After feeding nymph molts into adult tick. Adult male dies after mating and female dies after laying eggs completing the life cycle. The choice of hosts for each developmental stage can be different depending on the questing height. (Figure kindly provided by Martin Palus, with permission).

The host-seeking behaviour of tick is known as “questing” where they remain stationary on vegetation, extend their front legs and latch onto passing hosts. Tick have unique physiological mechanisms to detect and feed on their hosts. They can detect the presence of hosts from changes in temperature and carbon dioxide levels in the surrounding with the help of Haller’s organ situated on the front pair of their legs (Carr & Salgado, 2019; Süss, 2003).

Tick can feed on over 300 vertebrate species including mammals, birds, reptiles and humans (Anderson, 1991). Their mouthparts, adapted for blood-feeding, and saliva, which contains compounds to counteract host responses such as blood clotting, pain, itching, and inflammation, enable them to feed efficiently (Chitimia-Dobler et al., 2020; Sonenshine, 2005; Suppan et al., 2018). Each life stage has specific feeding duration and growth capacity. Larvae feed for 2-4 days and gain mass up to 20 times. Nymphs feed for 3-5 days and expand up to 40 times in size. Adult females feed for 7-10 days and gain mass up to 200 times (Sonenshine, 2005; Kahl

& Gray, 2023). Larvae typically feed on small mammals and nymph and adult feed on larger vertebrates like deer (Hillyard, 1996)(Figure 2). Adult female die post oviposition while males generally die after mating (Kahl, 1991; Süss, 2003).

Tick activity is closely linked to humidity, being most active when the relative humidity (RH) levels exceed 80-85% (Gray, 1991). Their questing peaks in the spring and autumn, aligning with optimal humidity levels (Cayol et al., 2017). Tick activity diminishes in hot, dry conditions or when there is high saturation deficit (SD) (Burri et al., 2011; Perret et al., 2004; Randolph & Storey, 1999). Excessively moist environments can be harmful, and tick do not quest during rain and wet conditions. The detachment from the host is influenced by host and environmental factors generally targeting optimal conditions for development to subsequent life stage (Kahl & Gray, 2023) .

Seasonal activity

Tick seasonal activity is influenced by climatic factors like temperature and humidity (Cayol et al., 2017).Tick is sensitive to environmental changes and have adaptations to increase their chances of survival in unfavourable conditions. Unfed tick undergo a behavioural diapause by halting their questing activity in anticipation of adverse winter conditions (Gray et al., 2016). Engorged tick undergoes a development diapause to improve survival through winter. Under unexpected environmental changes like cold snaps or droughts tick undergo quiescence with reduced metabolic activity for survival until favourable conditions return (Belozerov, 2009).

Typically, tick resume activity in the spring after the winter diapause. The timing of their blood-meal dictates their development. For example, spring-fed larvae molt into nymphs within a few months and those feeding late in the summer might delay molting until next summer, extending their life cycle (Kahl & Gray, 2023). In regions like Central and Northern Europe, molting predominantly occurs during the warmer months (Kahl & Gray, 2023). Tick activity pattern varies across Europe due to latitudinal and altitudinal differences. In Central Europe, nymph show a bimodal activity pattern, with two distinct peaks, one in May–June and another in September-October (Estrada-Peña et al., 2004). Meanwhile in Northern Europe, tick typically show single model of activity due to a cooler climate (Lindgren et al., 2000). Similarly, altitude affects tick activity. Nymph activity in lower elevation sites, show bimodal patterns and those in higher elevation show a single peak due to low temperature in high altitude areas (Bregnard et al., 2021).

1.3 Tick hosts

Tick are dependent on hosts for survival and dispersal over geographical areas (Sonenshine, 2005). Small mammals such as bank voles (*Myodes glareolus*), yellow-necked mice (*Apodemus flavicollis*), wood mice (*Apodemus sylvaticus*), and common shrews (*Sorex araneus*) are primary hosts for larvae and nymph (Achazi et al., 2011; Daniel et al., 2018). Many rodents in endemic areas exhibit both prenatal and postnatal TBEV transmission (Bakhvalova et al., 2009; Benda, 1958; Danielová & Holubová, 1991). Rodents usually have high tick aggregation and "20/80 " is associated with it- approximately 80% of tick feed on 20% of rodents (Esser et al., 2016). In natural ecosystems, these mammals sustain tick and act reservoirs of TBEV and this makes them suitable sentinel for TBEV monitoring (Achazi et al., 2011; Michelitsch et al., 2019).

Large vertebrates such as roe deer (*Capreolus capreolus*), fallow deer (*Dama dama*), and red deer (*Cervus elaphus*) are important hosts for adult tick (Imhoff et al., 2015). Habitats rich in deer are noted to have abundant tick populations (Gilbert et al., 2000; Jaenson et al., 2012; Tälleklint & Jaenson, 1998). Large vertebrates like deer including sheep and goats show detectable antibody response after TBEV infection (Kiffner et al., 2012; Klaus et al., 2012; Labuda et al., 1993). Cervids like roe deer, red deer, moose are distributed throughout Scandinavia and are used as sentinel for TBEV monitoring (Imhoff et al., 2015; Jaenson et al., 2012; Paulsen et al., 2020). The role of cervids in ecosystems and sustaining tick population has been discussed in many studies (Mysterud, 2000; Mysterud et al., 2014, 2018; Ruiz-Fons et al., 2012; Tagliapietra et al., 2011). In Scandinavian mountain regions, reindeer are abundant which have large habitat coverage from coastal to mountain regions (Paulsen et al., 2020; Sánchez Romano et al., 2019; Shakya et al., 2022).

Hosts, particularly birds may also serve as agents for dispersal of infected tick that are capable of starting an independent TBEV foci. Birds of species like dunnock (*Prunella modularis*), redstart (*Phoenicurus Phoenicurus*), song thrush (*Turdus philomelos*), mistle thrush (*Turdus viscivorus*), fieldfare (*Turdus pilaris*), common Blackbird (*Turdus merula*), willow warber (*Phylloscopus trochilus*), tree pipit (*Anthus trivialis*) were found to transport infected tick to Northern Norway (Hasle et al., 2009; Waldenström et al., 2007). Migratory birds often rest at specific sites during their seasonal migration, where they either may become infested with tick or drop off engorged tick (Hasle et al., 2009; Sándor et al., 2014; Waldenström et al., 2007; Wilhelmsson et al., 2020).

1.4 TBEV transmission

TBEV transmission routes

TBEV circulates within tick populations through several transmission routes that can be directly within tick lifecycle or by involving hosts (Havlíková et al., 2013).

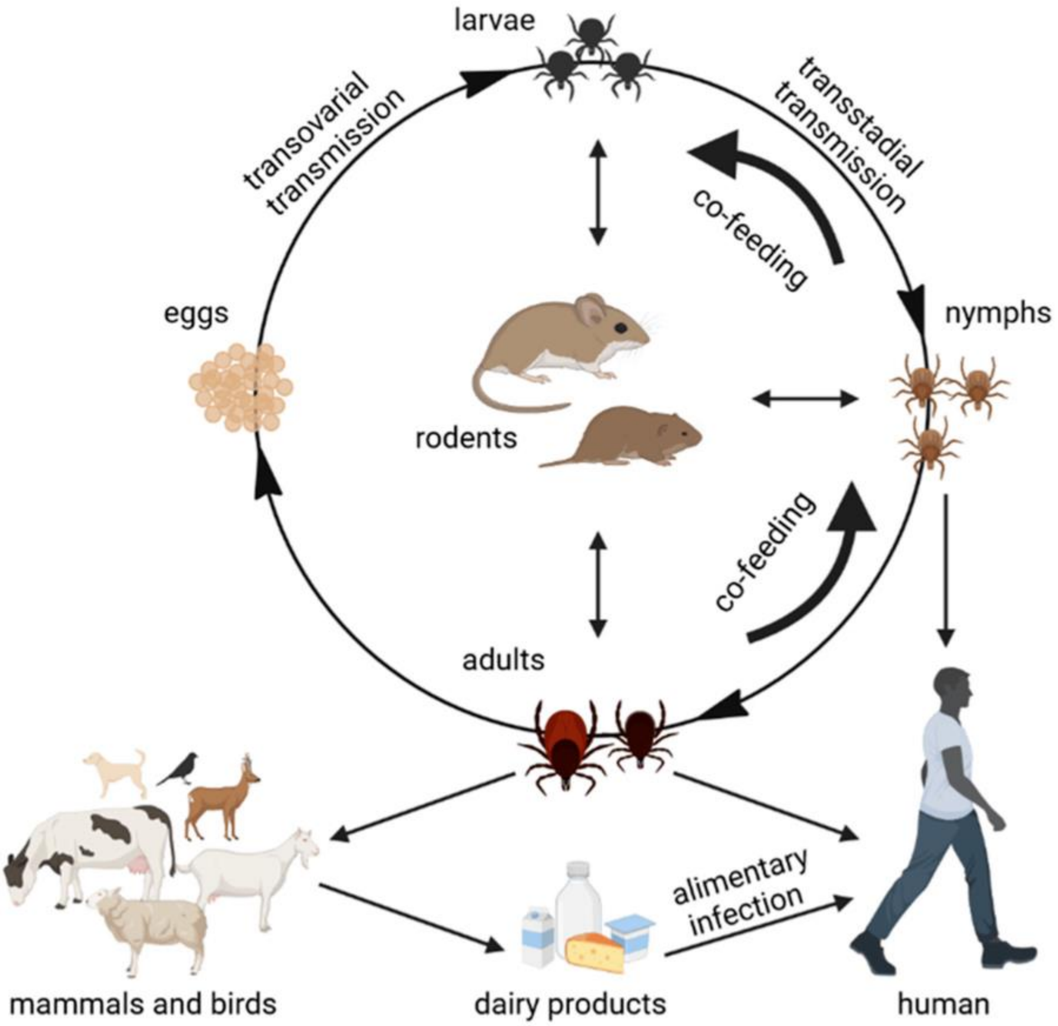

Figure 3: Modes of TBEV transmission in tick. Human infection occurs by infected tick bite or through the consumption of infected dairy products. Figure adapted from (Ličková et al., 2021).

TBEV transmission can occur through both viremic and non-viremic routes involving hosts. Viremic transmission can occur by blood-feeding on viremic hosts. Rodents from endemic areas were found to have considerable amount of viremia in their blood which supports their role as reservoir hosts for TBEV (Achazi et al., 2011; Bregnard et al., 2021). Non-viremic or cofeeding transmission occurs when larvae and nymph feed on the same host in proximity, where the virus moves from infected tick to uninfected tick using the host as a bridge for transmission (Labuda et al., 1993). Here, the tick itself is a reservoir of the virus and the host is merely a bridge for transmission (Figure 4). Larvae acquiring TBEV from an infected female through the egg is known as transovarial transmission. Once acquired, the TBEV can persist through all life stages. This is known as transstadial transmission (Figure 3) (Danielova et al., 2002; Nuttall & Labuda, 2003).

Nymphs tend to attract more larvae on specific host body regions like ears, underlining the importance of specific host body regions in co-feeding transmission (Esser et al., 2016; Randolph & Storey, 1999). Generally, climatic conditions are conducive for simultaneous questing of larvae and nymphs from spring to autumn in Europe (Burri et al., 2011; Cayol et al., 2017). The feeding synchrony can vary between years depending on annual variations in climate and/or host abundance. Rodent populations of the *Apodemus* and *Myodes* genera peak in summer or autumn in temperate European forests (Crespin et al., 2002; Pucek et al., 1993; Stenseth et al., 2002). Correspondingly, larvae and nymph activity peak in spring, and autumn (Gray et al., 2016; Perez et al., 2017; Randolph et al., 2000). Changes in the small mammal community structure (relative density and proportion of rodent species) over time can affect TBEV transmission. For example, tick burden and transmission-competence may differ between species, for example *Apodemus flavicollis* and *Myodes glareolus* (Dizij & Kurtenbach, 1995; Kurtenbach et al., 1995). The relative importance of these transmission pathways remains uncertain (Michelitsch et al., 2019).

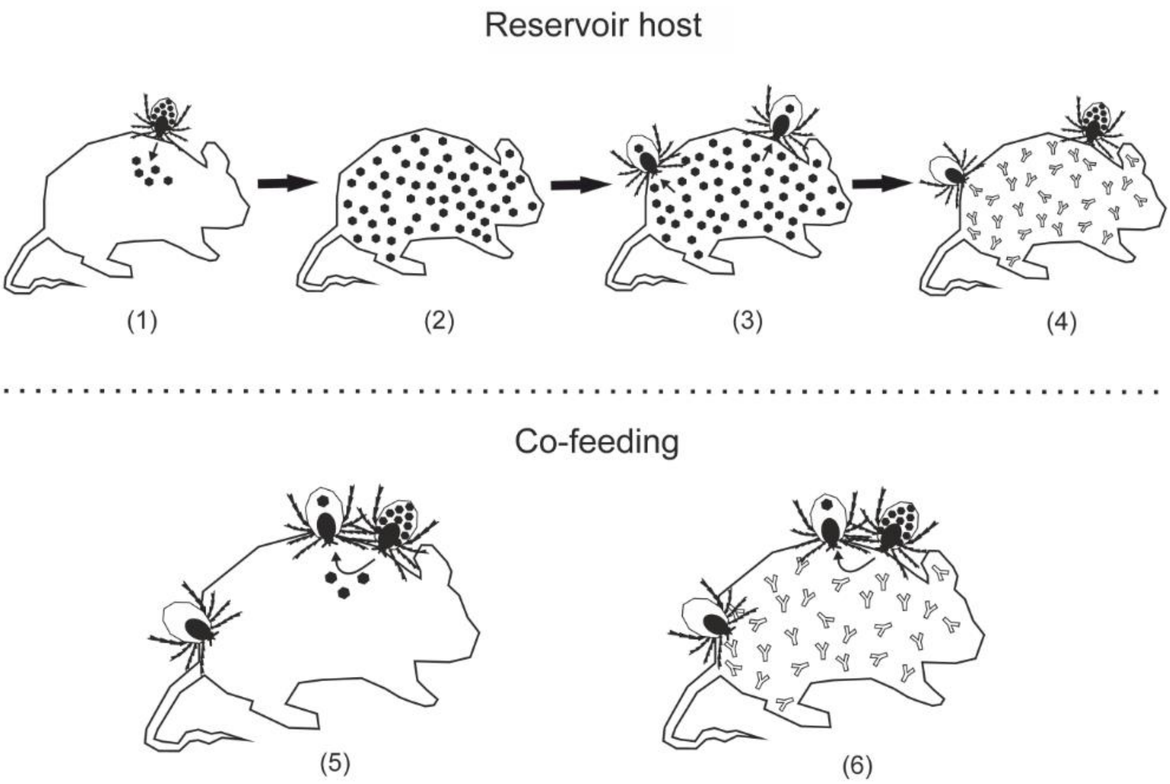

Figure 4: Reservoir hosts for TBEV. (1) An infected tick-bite transmits the virus to a host, (2) leading it to viremia, (3) uninfected tick acquires TBEV by consuming the blood of the viremic host, (4) this route of transmission is blocked by circulating antibodies as soon as the viremia is off, (5) co-feeding tick pass the TBEV among themselves without the viremic host using it as a transmission bridge (6) this can occur despite the host has antibodies against TBE. Figure adapted from (Michelitsch et al, 2019).

TBEV foci

Areas where TBEV-infected tick are regularly found over a number of years are known as "TBEV foci". In nature, a TBE focus can be "enzootic" or "endemic". A stable presence of circulation of the virus in tick and reservoir hosts causing minimal or no disease is called the enzootic mode. In endemic mode, enzootic circulation with regular infection in human and animals occurs (Süss, 2011). For TBEV foci to form and sustain, several conditions must apply including the introduction of virus by tick or hosts, optimal microclimatic conditions, sufficient reservoir hosts, and the virus's ability to multiply and endure in tick (Burri et al., 2011; Dobler et al., 2011). If these conditions are met, a TBEV focus can establish and persist autonomously (Dobler et al., 2011; Süss, 2003). The identification of TBEV foci can be done by screening tick, conducting serosurveys of humans and sentinel animals (Imhoff et al., 2015).

TBE foci generally have abundant tick and reservoir hosts with conducive microclimate and vegetation (Dobler et al., 2011). These foci can be dynamic with new foci emerging, or can become dormant or reactivated depending on conditions (Dobler et al., 2011; Lukan et al., 2010). For variation in TBEV prevalence over time, factors like food availability of rodent hosts, suitable microclimatic conditions play a major role (Crespin et al., 2002; Gray et al., 2016; Perez et al., 2017; Pucek et al., 1993; Randolph et al., 2000; Stenseth et al., 2002). Inter-annual variation in seed production (masting) by deciduous trees like beech and oaks were found to affect the density of vertebrate hosts (Bregnard et al., 2021). Increased seed and berry production were observed to trigger high rodent populations (Selås, 2006; Selås et al., 2019).

1.5 Scandinavia and climate change

Geography and vegetation

Scandinavia lies in northern Europe, encompassing Norway, Sweden, and Denmark. It has diverse climatic conditions and vegetation due to wide latitudinal coverage, central Scandinavian mountains, and influence of adjacent water bodies. The central mountain range separates Norway and Sweden, making central highland areas and lowland coastal areas. Southern Sweden and Denmark is mostly flat with a few low hills, and low-lying regions. The Gulf stream exerts a moderating influence on the Norwegian coastlines with maritime climate (Hurrell & Van Loon, 1997). The southern coastal regions including Denmark and parts of Norway and Sweden are influenced by the Skagerrak, Øresund and Kattegat straits. While the Skagerrak has relatively stable climatic conditions, Kattegat, situated between Jutland and Sweden's western coast, lies at the confluence of the North Sea and the Baltic Sea, influencing its weather pattern (Rosenberg et al., 1996). Additionally, the Swedish coastline along the Baltic Sea transitions from maritime to continental climate, with scanty rainfall (Christensen et al., 2022).

The northern regions of Scandinavia hosts worlds northernmost birch forests comprising of mountain birch (*Betula pubescens* subspecies c*zerepanovii*) (Kullman, 1997). The birch treeline marks the transition from boreal forest to alpine regions (Bandekar & Odland, 2017; Hallang et al., 2022; Kullman & Öberg, 2022). As one moves north or rises in elevation, the dense birch forests become shorter and sparser (Moen et al., 2004). With the exception for southern nemoral zone, most of Norway and Sweden lies within boreal region (Bandekar et al., 2020).While temperature is a key factor in determining the types of vegetation, other factors like topography and wind pattern may have more pronounced role (Bandekar & Odland, 2017). For instance, in the mountainous regions even if the temperature is relatively mild for the latitude, the landscape's elevation and steep terrain can create conditions more typical of a colder climate. Similarly, in certain coastal areas, strong winds from the sea can influence the types of plants that thrive there, even if the overall temperature is moderate. The southern and western parts of Scandinavia including Denmark, Southern Sweden and parts of Norway’s south coast, belong to nemoral zone characterized by deciduous forests with predominance of beech (*Fagus sylvatica*). The boreo-nemoral zone is a transitional zone that lies between the boreal zone and the nemoral zone. The boreo-nemoral zone covers many parts of southern and central Sweden and parts of eastern Norway with conifer forests comprising Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*) (Kullman, 1997).

Climate change impacts

Climate change in this context refers to the increase in the surface temperature of earth in past few decades primarily due to anthropogenic greenhouse gas emissions. These emissions mainly stem from activities like fossil fuel usage, land-use changes, and agriculture. According to the Intergovernmental Panel on Climate Change (IPCC), the average global temperatures have risen by 1.5°C above preindustrial times (1720–1800) (Field et al., 2014). The ‘climate’ refers to average atmospheric conditions of a place over a period of time while 'weather', refers to short-term like day-to-day variation in an area. There are considerable variations in the weather patterns over decades leading to climate change in the long term (Field et al., 2014). For instance, a few hot summer days in a place, like Oslo do not indicate climate change, but a decade of increasing summer temperature does.

The impacts of climate change in Scandinavia are of particular interest because of its wide latitudinal coverage, and colder ambient temperature which acts as a natural barrier for various vector-borne diseases (Van Oort et al., 2020). Northern areas and mountainous regions are more vulnerable to the impacts of climate change than southern and coastal regions due to factors like amplified warming due to snow cover and permafrost, and altered precipitation patterns (Rizzi et al., 2018; Thoman et al., 2022).

Changes in vegetation

Scandinavia has experienced on average a 1°C rise in annual temperature compared to the preindustrial times (Hanssen-Bauer et al., 2017; Lind & Kjellström, 2008; The Danish Government, 2008). Complementing this rise, an increase in precipitation has been reported from Norway and Sweden (Hanssen-Bauer et al., 2017; Lind & Kjellström, 2008). Due to changes in these abiotic factors, noticeable changes in the biotic communities have been observed as discussed in subsequent sections.

Changes in temperature and precipitation patterns have resulted in earlier onset of spring and delayed autumn in Scandinavia (Bird, 2017; Hanssen-Bauer et al., 2017; Norden, 2009; Räisänen & Alexandersson, 2003). This has extended the length of the vegetation period (GSL) and increased warmth by altering growing seasons and growing degree days (GDD) leading to changes in vegetation period (Chamberlain & Wolkovich, 2023).

Treelines, marking the upper boundary where trees can grow due to factors such as temperature, altitude, and climate, have moved up both latitudinally and altitudinally. Tree species have extended northward by 50 to 300 km and there is an increase in both canopy cover and vegetation density (Kullman, 2008). Birch treelines, indicative of climate suitable for plant growth, have migrated to areas previously characterized as tundra (Truong et al., 2007). This change is accompanied by the northward spread of thermophilus broadleaved trees like Downy Birch *(Betula pubescens*), Birch (*Betula pendula)*, Oak (*Quercus robur)*, Elm (*Ulmus glabra)*, Maple (*Acer platanoides)*, Alder (*Alnus glutinosa)*, and into previously colder areas (Kullman, 2008).

Phenological studies have observed earlier onset of natural events such as flowering and leafing (Menzel et al., 2006). For example, birch trees are beginning their pollen season earlier and birds are adjusting their migration and breeding times (Gregory et al., 2009; Norden, 2009).Tick are extending their range northward where northernmost viable population were found at Dønna approx 80 km north of Brønnøy (Hvidsten et al., 2020; Jore et al., 2011; Soleng et al., 2018). There is notable change in vegetation with dwarf-shrubs dominating over lichens (Vanneste et al., 2017). Bryn (2008) found that while forest expansion from 1959-1995 was largely due to reduced human and animal activity, climatic changes have played significant role since 1995, suggesting a potential for further forest migration. In central Norway, a shift in mountain vegetation over 22 years, marked by an increase in evergreens and herbs, has been related to increasing temperatures (Løkken et al., 2020). While climate change is a driving factor, land use changes also significantly influence these vegetation dynamics (Roessler et al., 2008).

Projections

The IPCC Representative Concentration Pathways (RCPs) outline future climate scenarios based on varying greenhouse gas levels, ranging from RCP2.6 (low), RCP4.5 (moderate) to RCP8.5 (high).

- **Temperature and precipitation projections**

Upcoming climatic shifts, especially in northern and high-altitude regions, are expected to intensify (Hanssen-Bauer et al., 2017; The Danish Government, 2008). Northern Europe may see summers extending by a month under RCP4.5 by mid-21st century (Ruosteenoja et al., 2020). Norway's temperature could rise up to 4.5°C by 2100, with north experiencing significant warming (Figure 5). Northern Sweden anticipates a 6°C temperature increase and 25% more precipitation, while Southern Sweden expects a 4°C rise in temperature and 11% more precipitation (Lind & Kjellström, 2008). The Baltic Sea northern part will also warm considerably (Christensen et al., 2022). A study on sea level changes in Denmark projects a significant rise in relative sea level in the Skagerrak-Kattegat Seas, exceeding 40 cm by the end of the twenty-first century. This rise, under the RCP8.5 scenario, will outpace the Fennoscandian post-glacial land-uplift (Su et al., 2021).

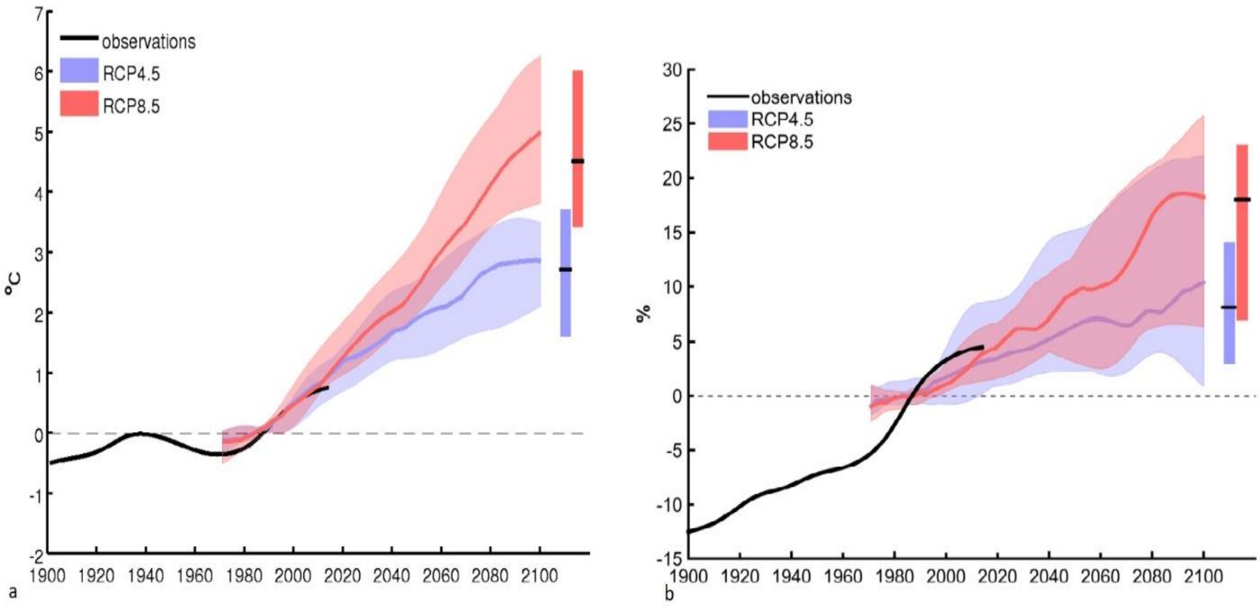

Figure 5: (a) Projections of temperature and (b) precipitation changes in Norway under different emission scenarios (with the reference period 1971-2000). Observed data from 1900-2014 in black curve while red and blue curves show median projections for RCP4.5 and RCP8.5. The RCP4.5 and RCP8.5 scenarios project an increase in temperature and precipitation by 2100. Figure adapted from (Hanssen-Bauer et al., 2017).

- **Vegetation and growing season projections**

The growing seasons are predicted to be longer and warmer between 2021 and 2050 (Hanssen-Bauer et al., 2017; Skaugen & Tveito, 2004)). By 2100, GSL in northern Norway could extend from current range of 30 to 180 days to 90 to 300 days driven by shorter snow-cover durations (Figure 6).Warmer seasons in northern Scandinavia may facilitate the northward migration of temperate species, increasing forest undergrowth (Bryn, 2008; Hallang et al., 2022; Langvall & Ottosson Löfvenius, 2021; Vanneste et al., 2017). The Swedish mountain treelines might rise by 233-667m altering mountain ecosystems (Moen et al., 2004). These vegetational shifts could impact tick populations positively (Alkishe et al., 2017; Ogden et al., 2021; Van Oort et al., 2020).

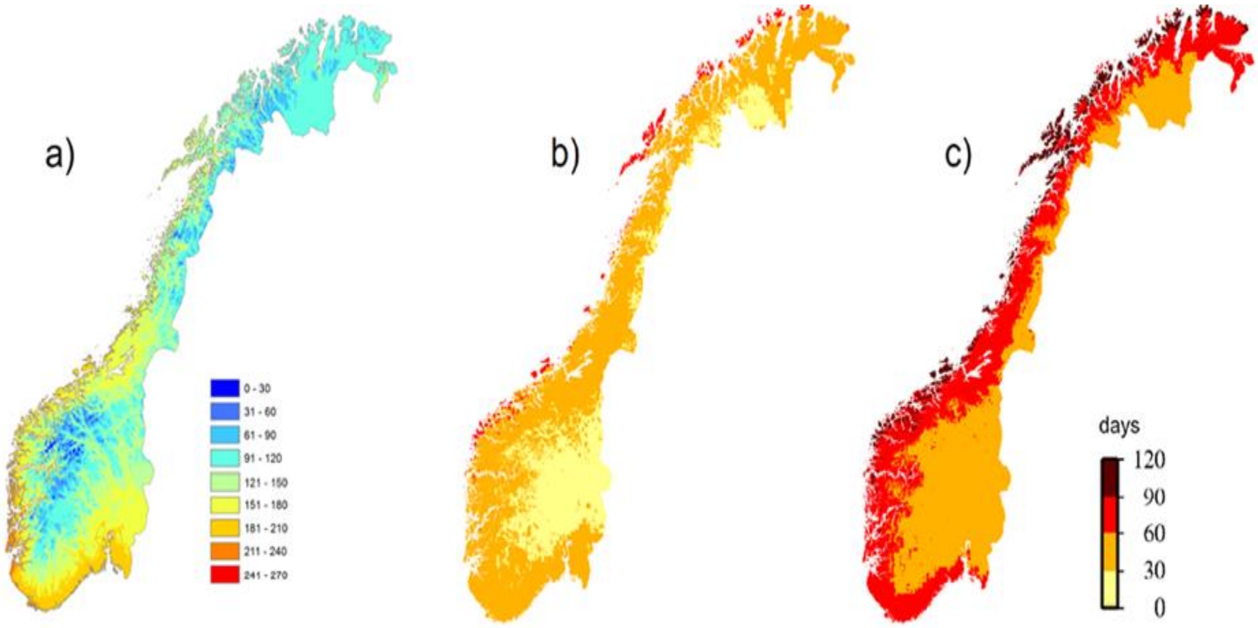

Figure 6: Historical and projected length of growing seasons in Norway. (a) During the reference period 1971-2000, the coast of Western Norway experiences the longest growing season (210-240 days) while mountainous regions have <60 days; an area of ~37,000 km^2 *has a growing season over six months. (b) For RCP4.5 by 2100, the growing season extends by one to two months for much of the country, and >2 months for north-western coastal regions, increasing the >6-month season area to 105,000* km^2*. (c) RCP8.5 predicts an even longer season, with the >6-month area expanding to 165,000* km^2 *by the end of the century. Figure adapted from (Hanssen-Bauer et al., 2017).*

1.6 Overview of TBEV studies from Scandinavia

1.6.1 Norway

Tick is predominantly found in coastal areas of Norway up to 66° N and at altitudes up to 1000 meters above sea level (De Pelsmaeker et al., 2021; Mehl, 1983; Soleng et al., 2018). TBEV-infected tick are reported, from coastal regions, from the Swedish border in the southeast to Brønnøy at 65.2°N (Soleng et al., 2018; Vikse et al., 2020). There is no notable tick presence in the inland mountain regions (Andreasen et al., 2020; Qviller et al., 2014; Soleng et al., 2018; Vikse et al., 2020). A positive moose sample from Steinkjer in central Norway represents the northernmost detection of a large TBEV seropositive animal in Norway (Paulsen et al., 2019).

The history of TBE in Norway officially started in 1997 when a TBE case caused by TBEV-EU subtype was reported from Tromøy island in Southern Norway (Skarpaas et al., 2004, 2006). A TBEV prevalence of 0.2% to 0.3% was found in tick near the site where the tick-bite occurred (Skarpaas et al., 2006). Although the first human TBE case was reported in 1997 from southern Norway, a serological evidence for its presence was reported much earlier from western Norway (Traavik, 1979). Suspensions of questing tick were injected into mice, and infected serum (from mice that showed clinical signs) were analysed. Five positive samples with a close serological relationship to the TBEV complex were detected showing circulation of TBEV or a related flavivirus in the western coast as early as around the 1970s (Traavik et al., 1978).

Human cases

Human cases occur from southern and south-eastern Norway in the counties of Agder, Vestfold and Telemark, and Viken (Figure 7) (Andreassen et al., 2012; MSIS, 2023; Vikse et al., 2020). The reported TBEV prevalence estimates in tick in these endemic regions ranges from 0.1 to 3.3% in nymphs and up to 20% in adult tick (Table 1). TBE incidence in humans show a seasonal pattern with most infections caused by tick bites from summer and autumn (MSIS, 2023).TBE has been notifiable to the Norwegian Surveillance System for Communicable Diseases (MSIS) since 1994.

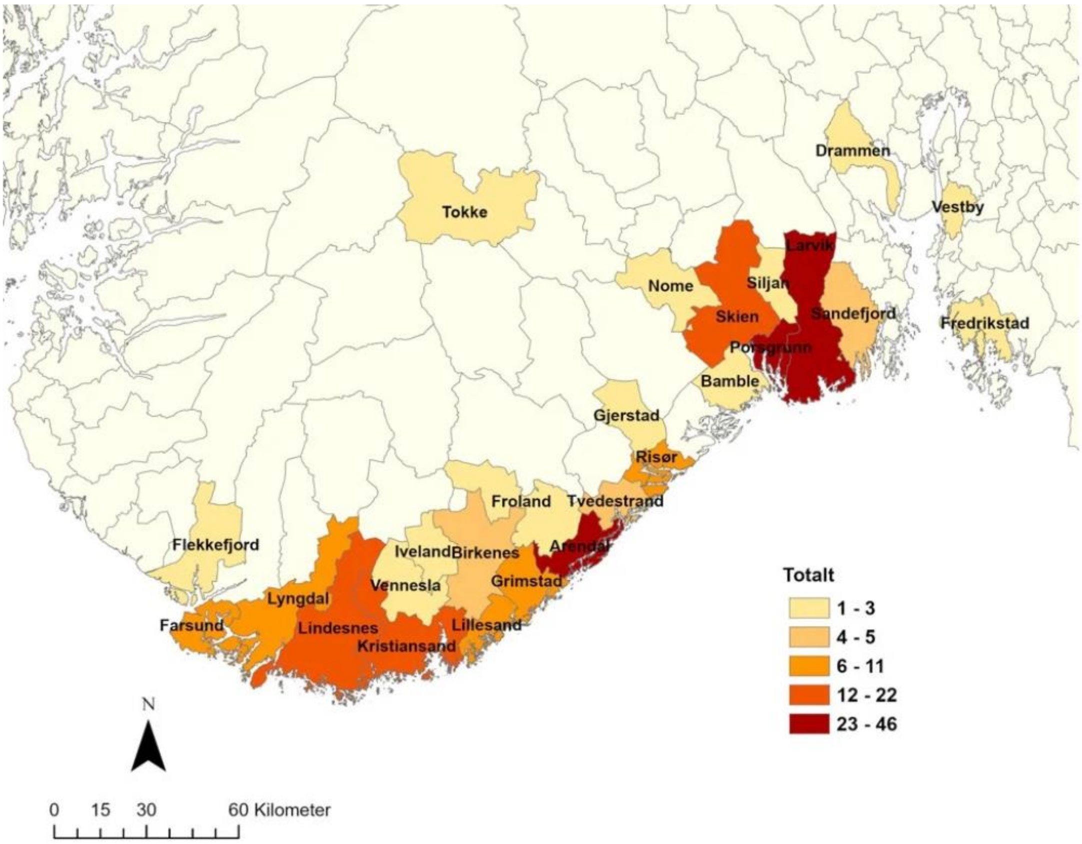

Figure 7: Total number of TBE reported cases in Norway by geographical area MSIS 1994-2021.

(adopted from https://www.fhi.no/en/in/smittevernveilederen/sykdommer-a-a/tick-borne-encephalitis--tbe-virus-infections/)

Antibodies against TBEV was detected in cervids ,cows, blood donors, suggests a regular TBE infection (Larsen et al., 2014; Paulsen et al., 2019, 2020). Presence of antibodies against TBEV in unvaccinated individuals may indicate past TBE infections (Larsen et al., 2014; Marvik et al., 2021). Endemic regions may have circulation of virulent strains causing symptomatic infections. In parts where TBEV has been detected in ticks, but no human cases have been reported, this may be due to viral loads too low to trigger symptomatic reaction or circulation of milder strains. The first Norwegian TBEV strain “Mandal 2009” was isolated from Norway which shared similarities with virulent Hypr strain suggesting circulation of a virulent strain in southern Norway (Asghar, 2014, 2017). Climate change is expected to make the inland and northern regions habitable for tick (Jore et al., 2014).

Table 1: Overview of human cases and TBEV prevalence in I. ricinus in Norway

Regions of Norway	**County**	**Reported human TBE cases**	***I.ricinus life stage***	**Prevalence range % (number of positive samples/total)**	**Reference**
Below arctic circle	Nordland	**No**	Nymphs	0.1-3.0	Soleng et al. 2018
		-	Adults	0-9.0	Soleng et al. 2018
Central	Trøndelag	**No**	Nymphs	0.1-0.41	Vikse et al. 2020; Paulsen et al. 2015; Soleng et al. 2018
		-	Adults	0-8.6	Vikse et al. 2020; Paulsen et al. 2015; Soleng et al. 2018
Central	Møre og Romsdal	**No**	Nymphs	0-0.6	Vikse et al. 2020
		-	Adults	0-4.7	Vikse et al. 2020; Paulsen et al. 2015
Central	Hordaland	**No**	Nymphs	0-1.3	Vikse et al. 2020
		-	Adults	0-15.4	Vikse et al. 2020
Central	Rogaland	**No**	Nymphs	0-3.5	Vikse et al. 2020
		-	Adults	0-20.6	Vikse et al. 2020
Southern	Agder				
	Vest-Agder	**Yes**	Nymphs	0.2-0.3	Skarpaas et al. 2006
	Vestfold og Telemark				
	- Aust-Agder	**Yes**	Nymphs	0.1-3.3	Vikse et al. 2020; Andreassen et al. 2012; Skarpaas et al. 2006; Sidorenko et al. 2018; Kjelland et al. 2018
		-	Adults	0-20	Vikse et al. 2020; Sidorenko et al. 2018
	- Vest-Agder	**Yes**	Nymphs	0-1.2	Vikse et al. 2020; Andreassen et al. 2012; Skarpaas et al. 2006; Kjelland et al. 2018
		-	Adults	0-20	Vikse et al. 2020; Sidorenko et al. 2018
	- Telemark	**Yes**	Nymphs	0-0.6	Vikse et al. 2020
		-	Adults	1.4-9.5	Vikse et al. 2020
South-eastern	Viken				
	- Østfold	**No**	Nymphs	0-0.4	Vikse et al. 2020, Larsen et al. 2014
		-	Adults	0-15	Vikse et al. 2020
	- Vestfold	**Yes**	Nymphs	0-0.9	Vikse et al. 2020
		-	Adults	[Data issue]	Vikse et al. 2020
	- Akershus	**No**	Nymphs	0-0.4	Vikse et al. 2020
	- Buskerud	**Yes**	Adults	0-8.6	Vikse et al. 2020

1.6.2 Sweden

TBE cases in Sweden have been reported from southern and central regions. National surveys show that cattle and sheep are exposed to TBEV in large parts of the country (Omazic, et al., 2023; Wallenhammar et al., 2020). Two tick species, *I. ricinus* and *I. persulcatus* are reported from northern Sweden along the Bothnian Bay (Jaenson & Wilhelmsson, 2019; Omazic, et al., 2023). It has been reviewed that prior to the early 1980s, the distribution of *I. ricinus,* was geographically confined to the south of the *Limes Norrlandicus* line in Central Sweden. This boundary marks the division between the southern nemoral zone and the northern boreal zone (Jaenson et al., 2012). The expansion of tick habitats in Sweden is attributed to change in host population mainly cervids like roe deer and climate (Jaenson et al., 2012). TBEV circulation in several areas south of central Sweden including Jönköping and western Sweden has been reported (Brinkley et al., 2008; Pettersson et al., 2014). Infected tick were found in passerine migratory birds like *Anthus trivialis* (tree pipit), *Turdus philomelos* (song thrush), *Phoenicurus phoenicurus* (redstart), *Erithacus rubecula* (robin) showing the role of migratory birds in dispersal of TBEV infected tick (Waldenström et al., 2007).

TBE is a long-standing disease in Sweden with regular human incidence (Figure 8). Most of the central and south-eastern Sweden is endemic to TBEV (Brinkley et al., 2008; Fält et al., 2006; Pettersson et al., 2014; Stjernberg et al., 2008; Wallenhammar et al., 2020). At Torö island south-east of Stockholm, the TBEV prevalence (MIR) was 0.5% in nymphs and 4.5% in adults (Pettersson et al., 2014).TBEV was commonly contracted by residents and visitors along Stockholm area, which is still a hotspot for TBEV infections (Pettersson et al., 2014; Public Health Agency of Sweden, 2023b). TBEV prevalence in tick within few locations near large water bodies from western Sweden varied between 0.1% and 0.4%, comparable to endemic areas (Andreassen et al., 2012; Brinkley et al., 2008; Dobler et al., 2011). Southernmost Sweden possibly has endemic regions as showed by retrospective serosurveys (Fält et al., 2006; Stjernberg et al., 2008; Svensson et al., 2021; Waldeck et al., 2022). Genetic data from a TBE patient in western Götaland and other TBEV sequences obtained from ticks and humans show the prevalence of the TBEV-EU subtype (Asghar et al., 2017; Brinkley et al., 2008; Elväng et al., 2011; Melik et al., 2007). TBE is a notifiable disease to the Public Health Agency of Sweden from 1969 (Holmgren & Forsgren, 1990; Lundkvist et al., 2011).

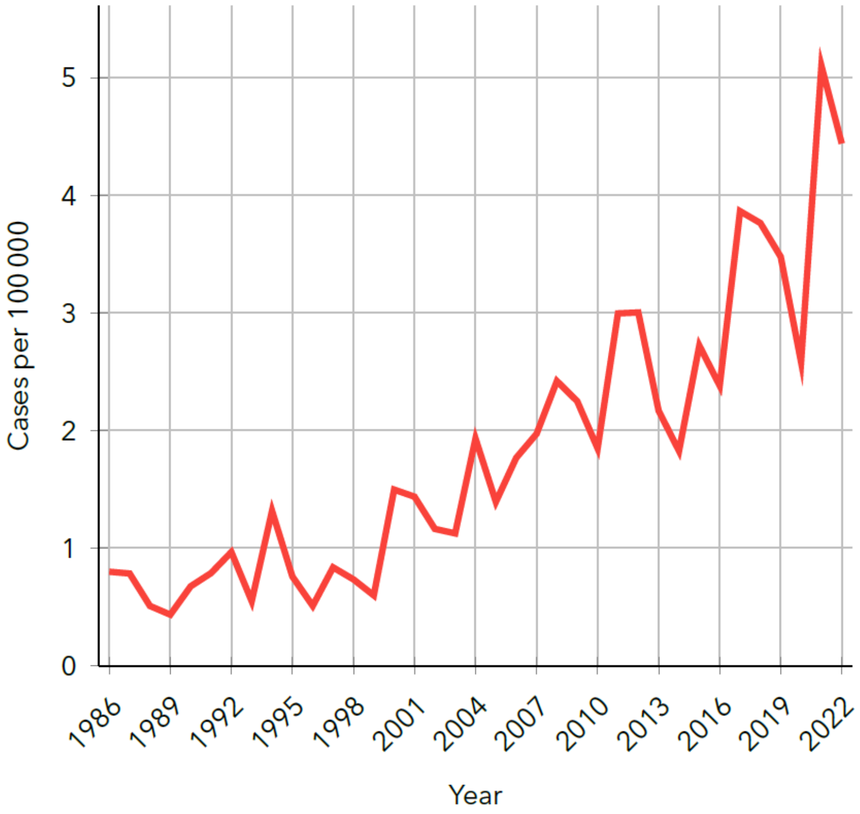

Figure 8: Incidence (per 100 000 inhabitants) of notified cases of tick-borne encephalitis in humans in Sweden 1986–2022. Figure adapted from (National Veterinary Institute of Sweden, SVA:s report series 89 1654-7098 2022)

TBEV strains like Toro 2003, Saringe 2009, JP-296 and JP-554, Habo and 1993-783 has been characterised from Sweden and belong to the TBEV-EU subtype (Asghar et al., 2017; Elväng et al., 2011; Haglund, et al., 2003, 2003; Melik et al., 2007; Paulsen et al., 2021). TBE cases are increasing in Sweden and warmer climate due to climate changes is expected to support tick expansion in previously inhospitable regions (Slunge et al., 2022).

1.6.3 Denmark

Although the Danish Island of Bornholm is a known endemic region, TBE infections in Denmark are relatively rare (Kristiansen, 2002). In 2009, two TBE clinical cases were reported from Northern Zealand and it was found to be caused by the European subtype of TBEV (Andersen, et al., 2019; Fomsgaard et al., 2009). In 2018, TBEV was contracted by two individuals from Northern Zealand (Tisvilde Hegn) (Andersen, et al., 2019). Strains isolated from there clustered with strains from Sweden and Norway (Andersen, Bestehorn, et al., 2019). By 2022, five hospitalized TBE cases have been reported from these areas (Statens Serum Institut, 2020). The genetic makeup of the TBEV strain from Bornholm, which historically mirrored strains from the Baltics, now resembled strains from Switzerland and Finland (Andersen, et al., 2019).

The geography of Denmark predominantly consists of lowlands surrounded by water bodies and connecting straits. TBEV prevalence in tick from several regions was suspected as long back as before in 2005 where roe deer tested positive for TBEV complex, but the results could not be confirmed due to the potential for cross-reactions with the Louping ill virus (Skarphédinsson et al., 2005). Later in 2019, presence of multiple TBEV positive sites in Denmark was confirmed with deer study was shown (Andersen, Larsen, et al., 2019).

1.7 Knowledge gaps

While many studies have examined TBEV prevalence in Scandinavia, there are knowledge gaps that need to be addressed. Given the impacts of ongoing global climatic changes and geographical range expansion of tick, surveillance of TBEV in regions where it already exists and in regions where it does not exist i.e., on both coastal and alpine regions is important. TBEV screening in tick allow direct detection of virus in its vector population which can potentially be transmitted to humans. This provides information about geographic distribution, prevalence rates, and changes in infection prevalence over time which is important for TBEV surveillance and diagnostics.

Detection and reporting of TBE cases is an inseparable part to the understanding of TBEV. TBEV-Eu often presents mild symptoms that can be overlooked. Despite widespread TBEV enzootic circulation along coastal regions, reporting of human cases from specific regions makes it important to explore this to gain a more comprehensive understanding on its impact on human health. The impact of climatic changes on virus distribution and evolution of is largely unknown. Development of sensitive detection method for TBEV in ticks which generally has a low viral load is important to properly gauge the disease's prevalence and impact.

1.8 Aims of the study

The objective of this thesis was to gain comprehensive understanding on TBEV in Scandinavia by gaining insights into TBEV distribution and diagnostics in the light of ongoing climatic changes.

The specific aims were:

1. Examine the distribution of TBEV infected tick in coastal lowland regions by analysing questing tick and inland mountainous regions using reindeer serum (Paper I and II),
2. To evaluate possible underreporting of TBE by analysing antibodies against TBEV in serum samples from patients with CNS symptoms (Paper III).
3. To establish a WGS method for two TBEV strains and perform bioinformatics and phylogenetics for understanding the strains (Paper IV).

2. TBEV detection and surveillance

Broadly, two groups of methods are used for TBEV detection and diagnosis: molecule based (those targeting the virus) and serology (those detecting the host's immune response to the virus).

2.1 Molecular methods

Polymerase chain reaction (PCR)

PCR is a widely used technique for TBEV detection due to its ability to amplify minute quantities of viral genetic material. PCR can be used to detect TBEV nucleic acids in tick homogenates, and in blood, cerebrospinal fluid and tissue samples (Ergunay et al., 2016; Paulsen et al., 2020). The E-gene is commonly targeted for virus specific envelope protein (Andreassen et al., 2012; Schwaiger & Cassinotti, 2003; Skarpaas et al., 2006). NS proteins and UTR regions are also targeted due to their conserved roles in virus replication and regulation for broader strain identification (Achazi et al., 2011; Brinkley et al., 2008; Schwaiger & Cassinotti, 2003). A common method is the RT real-time PCR where TBEV-RNA is reversely transcribed to complementary DNA followed by PCR (Andreassen et al., 2012; Brinkley et al., 2008; Schwaiger & Cassinotti, 2003). One-step PCR combines reverse transcription and PCR in the same buffer system (Brinkley et al., 2008; Ergunay et al., 2016; Gäumann et al., 2010; Patel et al., 2013). While this can enhance sensitivity, it carries a risk of false positives due to contamination during amplification (Ergunay et al., 2016). In Multiplex PCR, different sets of primers can be used for simultaneous amplification of several targets in a single reaction (Quick et al., 2017).

The amplified PCR fragments are often subjected to sequencing for confirmation to rule out the chances of false positives in the PCR reaction. Methods like sanger sequencing or pyrosequencing can be used depending on the target size. Sanger sequencing is a chain termination method where DNA synthesis stops when special nucleotides without a hydroxyl group at the 3' end are randomly incorporated. Pyrosequencing is based on the principle of 'sequencing by synthesis'. It detects pyrophosphate (PPi) released during DNA synthesis, generating a light signal that is proportional to the number of incorporated nucleotides (Ronaghi et al., 2007). Pyrosequencing is usually used for sequencing smaller fragments of DNA (Achazi et al., 2011; Ronaghi et al., 2007). Its limitations include short read lengths (100-500 base pairs), making the sequencing of larger fragments challenging. It requires precise interpretation of the generated light signal to avoid potential errors. Pyrosequencing has been used for confirmation of PCR positive TBEV pools in earlier studies (Achazi et al., 2011; Andreassen et al., 2012; Paulsen et al., 2015; Vikse et al., 2020).

Whole genome sequencing

Whole Genome Sequencing (WGS) retrieves whole genome sequences of an organism which allows comprehensive understanding of genetic diversity, evolution, and pathogenesis. A number of studies have successfully retrieved complete genome of TBEV-Eu strains (Agergaard et al., 2019; Asghar et al., 2017; Zakotnik et al., 2022). WGS comprises of several approaches like amplicon, shotgun, targeted or metagenomic sequencing. Next-generation sequencing (NGS) is a high-throughput method that can be complemented by bioinformatics tools for data processing (Dahui, 2019).

Direct TBEV detection in ticks and serum is difficult due to low viral load, which is a major challenge in TBEV genome studies (Gäumann et al., 2010; Vikse et al., 2020). A majority of WGS for TBEV strains from Europe have been developed by cultivating TBEV in cell culture samples to increase virus amount (Asghar et al., 2017; Jaaskelainen et al., 2010). A recent study utilized an amplicon-based strategy with specific overlapping primers to directly sequence the complete TBEV genome from clinical samples (Zakotnik et al., 2022).

2.2 Serological methods

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a widely used method for sero-screening of human and sentinel animals for TBEV surveillance (Klaus et al., 2014; Larsen et al., 2014; Marvik et al., 2021; Paulsen et al., 2020; Waldeck et al., 2022). Specific IgG antibodies against TBEV in blood samples are screened to see past TBE infection (Ergunay et al., 2016). The major limitation for this technique is that it may produce false negatives if conducted prematurely post-infection where antibodies have not reached to detectable levels in blood (Ergunay et al., 2016). False positives are often reported among flaviviruses due to cross-reactivity with other viruses of the same serogroup (Rathore & St. John, 2020; Stiasny et al., 2021).

Neutralisation Test (NT)

NT is used to detect TBEV infection by detecting neutralizing antibodies directed against TBEV (LitzbaNadine et al., 2014; Stiasny et al., 2021). It is often used as confirmatory test to validate specific antibodies detected through ELISA (Lamsal et al., 2023; Marvik et al., 2021; Paulsen et al., 2020). Plaque reduction neutralization test (PRNT) measures the reduction in viral plaques and is used to assess the neutralizing capability of antibodies against specific viruses (LitzbaNadine et al., 2014). The focus forming assay (FFA) is a specialized method for quantifying infectious viral particles that initiate the formation of localized foci in cell culture, particularly suited for viruses with focal infections (Lindqvist et al., 2018). The technical complexity and long procedures often limit extensive screening using SNT compared to the ELISA tests.

Immunofluorescence Assay (IFA)

IFA is a laboratory technique that uses antibodies tagged with a fluorescent dye to detect specific viral antigens in a sample to identify viral infection (Girl et al., 2021; Kvapil et al., 2021). It combines a serum sample with flavivirus-infected cell culture to detect specific antibodies. IFA technique can directly detect antigens using fluorescently labelled antibodies. Its indirect variant (IIFA) uses secondary antibodies with a fluorescent label to detect specific antibodies in a sample (Shakya et al., 2022). IFA requires additional diagnostic tests for definitive flavivirus confirmation due to potential cross reaction with other flaviviruses (Girl et al., 2021). Therefore, IFA is often employed as a confirmatory or complementary test for visualisation and to provide confidence in the results (Kvapil et al., 2021).

2.3 Surveillance of TBEV

Surveillance of TBEV involves monitoring tick populations and their hosts, as well as human cases of TBE. This includes:

Estimating TBEV prevalence in tick population

Tick screening is an important method to estimate TBEV prevalence in a tick population. This method involves collecting ticks by flagging or dragging and screening ticks with molecular methods (Estrada-Peña et al., 2013). It is used for the identification of TBEV focus (Agergaard et al., 2019; Pettersson et al., 2014; Soleng et al., 2018; Vikse et al., 2020). This can be expensive and labour-intensive and the detection of TBEV in tick doesn't inherently signal an immediate threat to humans, as the infection risk is also influenced by human activities, tick density and viral load in the tick. While tick screening can show viral circulation and maintenance in tick populations, it is often complemented with other surveillance methods like human and sentinel studies for identification of TBE endemic foci (Gaëlle Gonzalez et al., 2022; Larsen et al., 2014; Stjernberg et al., 2008).

Sentinel animal surveillance

Sentinel animal surveillance involves identifying previous TBEV infection in animals (like deer, moose, goat, cow, sheep and various rodent species) that are often bitten by tick (Achazi et al., 2011; Imhoff et al., 2015; Klaus et al., 2012). Monitoring these "sentinel" animals can provide information about the status of enzootic circulation of TBEV in tick populations in a particular area. This approach is non-invasive, often more cost-effective than monitoring humans, and gives insights into large areas. However, it may underestimate the actual spread of TBEV since it doesn't cover all susceptible animal species (Imhoff et al., 2015). Therefore, it is usually paired with other methods such as human case reports and TBEV prevalence estimation in tick (Paulsen et al., 2020).

Human case reporting

Human case reporting involves the systematic recording and tracking of TBE infections in individuals. It plays a significant role in understanding the spread and public health impact of TBE, and it can be carried out retrospectively or prospectively (Fält et al., 2006; Larsen et al., 2014; Marvik et al., 2021; Waldeck et al., 2022). . By maintaining a record of TBE cases, health authorities can identify patterns, recognize high-risk regions, and evaluate the effectiveness of disease control strategies (Lindgren et al., 2000, 2012; Slunge et al., 2022). Data from animal surveillance along with human cases provide a comprehensive understanding of TBEV transmission and epidemiology.

3. Results and discussion

The geographic spread of Tick-borne encephalitis virus (TBEV) is a concern (Heyman et al., 2010; Slunge et al., 2022).TBEV may cause infection with symptoms ranging from subclinical to severe neurological disease. Ongoing climate change can influence tick distribution and TBEV infection rates in Scandinavia, potentially escalating the number of TBE cases. This PhD thesis aimed to investigate the epidemiology of TBEV in Scandinavia by studying tick vectors, sentinel animals and diagnostic methods. The study was conducted in four parts: Paper I and Paper II were aimed to understand the spread across Scandinavia and Paper III and IV were designed improve TBEV diagnostics.

3.1 Distribution TBEV infected tick in Scandinavia

The *I. ricinus,* vector for TBEV-Eu is widespread in Scandinavia (Kjær et al., 2019). TBEV was detected in 20 of the 50 collection sites, with overall prevalence of 0.1% in Norway and Denmark and 0.2% in Sweden in nymphs (Paper I). This finding is in line with previous studies which have confirmed TBEV circulation through tick screenings, serosurveys and sentinel studies (Agergaard et al., 2019; Andersen, et al., 2019; Andreassen et al., 2012; Fält et al., 2006; Larsen et al., 2014; Marvik et al., 2021; Paulsen et al., 2020; Stjernberg et al., 2008; Vikse et al., 2020; Waldeck et al., 2022). The ØKS region in Scandinavia is significant due its high population and regional cooperation (Agder fylkeskommune, 2021). Notably, several TBEV positive sites (Paper 1) corresponded to reported human TBE cases. Earlier, TBE cases occurred in known endemic foci in southern Norway (Andreassen et al., 2012). Recent trends show an expanding range of the foci from southern to south-eastern regions in Norway (MSIS 2022). This underscore expanding TBE endemic regions in southern Norway. Sentinel studies and tick prevalence studies suggested enzootic circulation of TBEV in these areas (Paulsen et al., 2020, Vikse et al., 2020).

The overall estimated TBEV prevalence was 0.1% (29/2957) (Paper I), indicating a low overall prevalence. Certain regions in Norway and Sweden, showed prevalence above 0.5%, suggesting an increased risk of TBEV transmission. At two locations on either side of the Oslofjord, the TBEV prevalence was higher (0.7%, and 0.5%) than previously reported (Vikse et al., 2020). The coastline around the Oslo fjord is among the most populated areas in Norway. There have been TBEV studies on blood donors and human cases from south-eastern Norway in the last five years (Larsen et al., 2014; Marvik et al., 2021; MSIS, 2023). In 2022, 68 cases of TBEV infection originated from tick bitten persons in Agder, Viken, Vestfold, and Telemark counties (MSIS, 2023).

TBE cases in Sweden doubled from 2013 to 2022, despite increased vaccinations (Public Health Agency of Sweden, 2023a). TBE infections have been consistent in Sweden for many years, mainly around Stockholm, region adjacent to lakes such as Vänern, and along the southern coastal areas (Kjær et al., 2023; Public Health Agency of Sweden, 2023b, 2023a). This confirms presence of TBEV in these areas. The findings from paper I reinforces that TBEV is prevalent in tick in several locations along Skåne, Kronoberg, Jönköping, Blekinge and Kalmar counties. Antibodies against TBEV were reported from milk from cows and goats from several dairy farms from Sweden underlining a continued risk to individuals who are exposed to tick in these areas (Omazic, et al., 2023; Wallenhammar et al., 2020). Supporting this finding, Waldeck et al., (2023) reported an increase in human TBE cases compared to previous data from retrospective studies. Thus, the number of TBE cases from the endemic regions is increasing (Public Health Agency of Sweden, 2023a). These retrospective studies have found antibodies against TBE in human serum samples from southern regions (Fält et al., 2006; Pettersson et al., 2014; Stjernberg et al., 2008; Svensson et al., 2021; Waldeck et al., 2023; Waldenström et al., 2007). The presence of these antibodies is a strong indication that TBE infections have occurred in these regions.

Interestingly, there is lack of comprehensive understanding as to why TBE cases were not reported earlier from these southern regions. One explanation is the introduction of TBEV into previously unaffected regions. Due to its proximity to the southeastern coast of Sweden, this region acts as a corridor for migratory birds, which could potentially carry tick from other areas thereby introducing TBEV to new environments (Waldenström, et al., 2007; Wilhelmsson et al., 2020).

In Denmark, Paper I identified 12 TBEV positive sites, where seven were from Jutland, three from Zealand and two from Bornholm and Falster each (Paper I, Figure1). The island of Bornholm is a long identified TBE endemic region in Denmark, and human cases are regularly reported from here. The risk areas for TBE in Denmark are considered Bornholm and forested areas in North Zealand (specifically Tisvilde, Asserbo, and Hareskoven) (Andersen, et al., 2019; Statens Serum Institut, 2020). A new TBEV micro focus was discovered in Denmark after several infections near a nature playground, with evidence suggesting the virus was brought by migratory birds and spread locally (Agergaard et al., 2019; Statens Serum Institut, 2023). In 2020 and 2021, all reported TBE infections in Denmark were locally acquired, with North Zealand emerging as a hotspot (Statens Serum Institut, 2023). Rising temperatures and deer presence may contribute to the virus's spread in Denmark, while in Sweden, climate change and changes in human behaviour are considered as factors (Jaenson et al., 2018; Jaenson et al., 2012; Statens Serum Institut, 2023). Sentinel studies have historically reported the presence of TBE antibodies in roe deer sera in Denmark. TBE seropositive roe deer were reported from various parts of Denmark as early as 2005 and in 2015, 8.7% and 6.9%, respectively (Skarphédinsson et al., 2005; Andersen, Larsen, et al., 2019). Our study, along with previous studies, suggests that TBEV is enzootic in various regions of Denmark (Agergaard et al., 2019; Andersen, Bestehorn, et al., 2019; Andersen, Larsen, et al., 2019; Fomsgaard et al., 2013; Skarphédinsson et al., 2005).

The impact of distinct geographical and ecological landscapes of Scandinavia on TBEV distribution is largely unknown. Denmark is primarily characterized by mostly sandy, low-elevation topography, predominantly used for agriculture (about 75% of the landmass). Norway and Sweden have extensive forested areas (over 80%) along with central mountain ranges in both coastal and inland regions. These varying geographical features could have a significant impact on tick populations and circulation of TBEV in these regions. While Denmark's flat agricultural landscape may offer different habitat conditions for tick and their hosts compared to forested terrains of Sweden and Norway, the exact effects of these topographical differences on distribution and behaviour of tick and hosts, and consequently on TBEV circulation are not fully understood. Moreover, factors such as wind direction and climatic conditions in Skagerrak and Kattegat remains yet to be investigated.

Inland highlands and northern areas

None of the reindeers tested in Paper II were positive for antibodies against TBEV. This is likely due to their habitat preferences in higher altitude areas where tick are rare or absent (Hvidsten et al, 2020, Qviller et al., 2014). Monitoring these regions is important as a baseline for detecting potential shifts in TBEV distribution due to climate change (Figure 5,6) (Holzmann et al., 2009; Lukan et al., 2010; Tokarevich et al., 2017; Tokarevich et al., 2011).

Studies have shown that increasing altitude limits the questing period and development rates of the tick and TBEV, probably due to climatic effects (Materna et al., 2005; Qviller et al., 2014).

Shift of TBE foci to higher altitude in Czech Republic has been reported showing that increase in tick density in higher altitude regions can support enzootic cycles of TBE (Daniel et al., 2003). Factors such as rising temperatures and changing humidity levels can create environments more conducive to tick survival and expansion (Alkishe et al., 2017) .

In similar studies using other cervids like moose, red deer, roe deer as sentinels have reported prevalence of TBEV in areas which overlaps with reindeer foraging areas (Paulsen et al., 2020). Moose and roe deer also forage in areas outside the reindeer foraging range and it could be there that they are exposed to tick and TBEV. This study supports the importance of considering a variety of wildlife hosts and environmental factors in tick studies (Imhoff et al., 2015). The samples analyzed in this study were collected in 2013-2014 and the conditions might have evolved with changing climatic conditions. Therefore, while coastal and lowland areas continue to report higher TBEV prevalence, our study suggests that mountainous regions appear less affected.

3.2 Climate, tick and TBEV in Scandinavia

Tick was collected simultaneously across the three countries within six weeks to minimise the impact of seasonal factors. In paper I, we showed that TBEV was detected in 20 of 50 analysed sites. Currently, TBE is limited to specific regions in Scandinavia, predominantly in southern Norway and Sweden as well as in Bornholm and Zealand in Denmark. A region-specific correlation between the TBEV prevalence and relative humidity (RH) was observed. Given the evolving climate conditions, TBE may emerge into new areas where it currently is absent (Ogden et al., 2021; Van Oort et al., 2020).

Microclimate and TBEV

Climate change is anticipated to bring more humid conditions in Scandinavia, potentially enhancing the transmission of TBEV (Alkishe et al., 2017; Hanssen-Bauer et al., 2017; Ruosteenoja et al., 2020). The circulation of TBEV in nature is shaped by various abiotic and biotic factors (Sirotkin & Korenberg, 2019). Results from paper I show the influence of RH on TBEV prevalence in tick populations suggesting that RH plays an important role in shaping TBEV dynamics. This is in accordance to earlier studies which suggested that RH, modulated by seasonal and local climatic changes, has the major influence on TBEV prevalence in tick (Danielová, 1990; Danielová et al., 1983). In a previous study from endemic regions of southern

Norway a correlation between TBEV prevalence and high humidity and low saturation deficit was shown (Andreassen et al., 2012).

The effect of humidity on TBEV prevalence are shown to be regional specific. The different humidity levels correlate with TBEV prevalence in southern inland regions of Sweden with a continental climate and the Oslofjord area with coastal climate. This suggests that the transmission dynamics of TBEV may be locally specific and consistently linked to humidity. In Denmark, no clear association between climate TBEV positive sites across the country was observed. A limitation of this study is that the data from the nearest meteorological stations were used. These might not fully capture the local microclimatic conditions influencing the tick’s life conditions (Estrada-Peña et al., 2004; Randolph & Storey, 1999). Additionally, the conclusions of this study are drawn from a limited number of positive sites in Norway and Sweden. A higher number of ticks collected at each site including larger geographic sampling areas might provide a more comprehensive understanding of the relationship between RH and TBEV prevalence.

Changes in microclimate due to longer seasons are associated with higher TBEV circulation in tick and rodents. This might be caused by changes in human activity and less grazing animals leading to denser vegetation and a more humid microclimate. Longer season results in higher abundance of larva and nymphs creating opportunities for TBEV transmission and maintenance within the tick and rodent population (Nah et al., 2020). Increasing humidity leads to a higher ratio of larval to nymphal tick feeding on rodents, affecting the seasonal and geographical transmission pattern of tick-borne disease (Randolph & Storey, 1999). A study reported the role of specific environmental conditions mainly humidity on increased TBE cases in Örebro County, Sweden, from 2010 to 2021(Kjær et al., 2023). This is supported by the correlation between number of nymphs and TBE cases, emphasizing the impact of tick abundance on TBE incidence (Borde et al., 2021).

Rising temperatures and humidity may increase the viral load of TBEV within the tick (Daniel et al., 2018; Danielová et al., 1983; Lindblom et al., 2014). Studies have reported that TBEV may replicate more rapidly in tick during feeding compared to when

they are unfed (Daniel et al., 2018; Lindblom et al., 2014). During this process, the virus replication may increase due to ingestion of the warm blood. Warmer temperatures in summer and autumn may also promote increased virus replication in tick, increasing the risk of contracting TBE from tick bites (Daniel et al., 2018; Danielova 1983). The viral load may influence the severity of symptoms, a person bitten by a tick with a high viral load experienced symptoms, while another bitten by a tick with a low viral load remained asymptomatic (Lindblom et al., 2014). We hypothesised that increasing temperatures and humidity may lead to higher TBE viral load in tick. If the virus is more prevalent during autumn compared to spring, this suggests that increasing temperatures and humidity could lead to a higher number of TBE cases with symptoms. Although, there may be several other confounding biological factors, the effect of climate on viral load is supported.

In northern Europe, tick habitats are expanding due to climate and environmental changes, prolonged growing seasons, and changes in vegetation patterns (Gray et al., 2009; Hvidsten et al., 2020; Jaenson & Lindgren, 2011; Lindgren et al., 2000; Lindquist & Vapalahti, 2008; Qviller et al., 2014). These changes have also led to an increase in hosts like rodents and deer, contributing to tick proliferation (Achazi et al., 2011; Hauser et al., 2018; Hedenås et al., 2016; Jaenson et al., 2012; Selås, 2006; Selås et al., 2019; Vanneste et al., 2017). Recently increase in *I. persulcatus* in its sympatric region with *I.ricinus* was observed in northern Sweden (Omazic, et al., 2023). This suggests a potential for changes in TBEV dynamics in the region (Belova et al., 2023; Kovalev et al., 2016).

3.3 Challenges in addressing TBEV

True TBE incidence

The prevalence of TBE infections in Europe is probably higher than currently known (Kunze et al., 2022; Schley et al., 2023). In our study, we detected one seropositive TBE case among 137 hospitalized cases with CNS symptoms with unknown cause. This suggests that TBEV could be overlooked.

A recent report suggests that the actual number of TBE cases in Scandinavia is higher than currently diagnosed or reported (Waldeck et al., 2022). The findings in Paper I, is in accordance with previous studies identifying TBEV foci at multiple locations (Paulsen et al., 2020; Vikse et al., 2020). In a comprehensive study, TBEV was found to circulate in areas with high tick density along the Norwegian coastline from Østfold to Nordland County. The findings showed an overall TBEV prevalence of 0.3% in nymphs and 4.3% in adults (Vikse et al., 2020). This suggests a broader distribution of TBEV than previously understood. Similar findings from

Germany and Sweden supports that underdiagnosis are more common than previously known (Haglund, Settergren, et al., 2003; Haglund, Vene, et al., 2003; Schley et al., 2023).

In southern Norway, antibodies against TBEV have been detected in unvaccinated blood donors implying the existence of subclinical or asymptomatic infections (Larsen et al., 2014; Marvik et al., 2021). Likewise, TBE antibodies were retrospectively detected in hospitalised cases, implying missed or undiagnosed TBE cases in studies from southern Sweden (Fält et al., 2006; Pettersson et al., 2014; Stjernberg et al., 2008; Svensson et al., 2021; Waldeck et al., 2023; Waldenström et al., 2007). Additionally, in Sweden Lindbolm *et al*., (2014) identified tick bitten individuals with TBE antibodies, indicating asymptomatic infections. In Denmark, TBEV circulation is confirmed in many regions, however, human cases are rare, a situation similar to Norway (Fomsgaard, 2020; MSIS, 2023; Vikse et al., 2020).

Diagnosing TBE can be challenging due to the non-specific nature of the initial symptoms and is typically based on detecting specific IgM antibodies in serum and cerebrospinal fluid (CSF). Once the antibodies develop, the viral genome cannot be detected, making early detection of TBE difficult. The fact that our single Norwegian TBE case was not reported to the MSIS system, suggests loopholes in the current reporting system. This gap can be attributed to various factors like communication problems between the laboratory and healthcare providers, misunderstood responsibilities, or unclear reporting protocols. Several other factors may contribute to underreporting, such non-mandatory reporting of TBE cases (as in Denmark), less virulent virus strains, low viral loads in infected tick, or a potential lack of awareness among healthcare providers and the general public (Schley et al., 2023). The focus of Paper III was on hospitalized CNS cases in Oslo, where residents frequently visit endemic areas for recreational purposes. Expanding this study by including more regions, including endemic areas, and larger sample sizes, would provide a more comprehensive picture of TBE incidence especially in cases with CNS symptoms of unknown origin.

Genetic changes and phylogenetics

In Paper IV, we established a method for WGS of European TBEV by using two cultured strains: Hochostervitz and 1993/783. The whole polyprotein sequence was retrieved, and phylogenetic analysis showed that Hochostervitz is genetically related to strains endemic in South Korea, while the 1993/783 is related to strains from the Netherlands (Paper IV, Figure I). The introduction of TBEV to new suitable areas by hosts, like cervids and birds, is an important factor for tick dispersal (Hasle et al., 2009; Jaenson et al., 2012; Paulsen et al., 2020; Waldeck et al., 2022; Wilhelmsson et al., 2020). Due to the geographical distance, migratory birds possibly introduced TBEV-Eu strains into regions like South Korea, where the TBEV-Fe subtype is more common. The involvement of *Ixodes tick*, acquired by migratory birds or potentially dispersed through international trade routes involving rodent reservoirs, suggests complex mechanisms of virus distribution (Carpi et al., 2008; Demina et al., 2010; Klaus et al., 2016; Nuttall & Labuda, 2003; Waldenström et al., 2007; Yun et al., 2011). Additionally, the analysis indicates that the 1993/783 strain clusters with strains from diverse European locations, indicating that TBE variants are not geographically confined as has been observed by other studies (Grubaugh et al., 2019; Heinze et al., 2012).

In Paper IV, we identified 52 ambiguous nucleotides in the TBEV Hochosterwitz and eight in the 1993/783 (Supplementary table 1, Paper IV). A potential source to the ambiguities within the polyprotein could be due to errors during genome replication, technical errors, such as PCR or primer induced errors or errors due to cultivation passages. In our study, the Hochosterwitz strain exhibits genetic variation in the whole polyprotein with similar percentage across the different fragments. These variations may affect interactions with host cells and their immune system (Kofler et al., 2002). The 1993/783 strain shows genetic variations in non-structural proteins that may affect viral replication and immune evasion (Pulkkinen et al., 2018). The observed differences between the Hochosterwitz strain, isolated from a tick, and the 1993/783 strain, isolated from a human, could stem from their distinct origins or cultivation histories (Helmová et al., 2020; Henningsson et al., 2016). The Hochosterwitz strain exhibits higher genomic diversity with four transition and four transversion mutations. It underwent two passages in mouse brain and eight passages in Vero E6. In the 1993/783 strain, which was initially passaged once in suckling baby mice and three times in Vero E6 cells, there were five amino acid changes. These mutations, 3 transitions and 2 transversions, are all in the non-structural regions regulating the expression and replication of the virus. One ambiguous mutation was related to primer error in the Hochosterwitz strain (Supplementary table 1, Paper IV). Among the other 52 mutations, most of them coded for the same amino acid. However, eight of these 52 mutations were amino acid changes that introduced an amino acid with different function. A consistent percentage of ambiguities across different fragments of the whole polyprotein suggests that these are inherited during the RNA replication of the virus (Supplementary table 1, Paper IV). If the ambiguous percentage were randomly distributed in the polyprotein at different percentages, it is more likely to be PCR amplification errors or other random mistakes. A plausible explanation to the evenly distributed errors is the genetic variability within the virus population, indicative of the virus adaptive strategies as a response to different environmental pressures, host interactions or possible quasispecies of the virus(Helmová et al., 2020; Henningsson et al., 2016; Kofler et al., 2002; Pulkkinen et al., 2018; Schneider & Roossinck, 2001). However, we cannot exclude the possibility that the sequence ambiguities arise from mutations that accumulated during *in vitro* passage of the virus in the absence of selection pressure.

3.4 Methodological considerations

This study can add knowledge in understanding the distribution and genetic variations of TBEV, underscoring the virus adaptability and potential for environmental adjustments. But it has some limitations, including a limited number of samples, study locations and sequenced strains, that may limit the generalizability of the findings.

In Paper I, a consistent methodology for tick collection was important for obtaining comparable results. The study collected nymphs; the most abundant life stage responsible for TBE infections. This approach of excluding adults could potentially lead to underestimation of actual prevalence of TBEV in tick, given a higher infection rates in adult tick (Vikse et al., 2020). Nonetheless, our study represents an improvement in consistency and facilitates a better comparative analysis than previous studies with varied pooling methods and life stages (Pettersson et al., 2014).

Estimating TBEV prevalence in tick is complex. Paper I followed a TBEV estimation approach where one positive pool in 74 pools analysed, with a pool size of 10, indicates a TBEV positive site (Andreassen et al., 2012; Ebert et al., 2010). However, since only 60 tick pools from each site were analysed, there is a possibility of underestimating the prevalence.

The discrepancy observed in Paper II and III, where samples tested positive in ELISA and negative in Serum Neutralization Test (SNT), can be explained by two main factors related to the characteristics of flavivirus diagnostics. ELISA is known for its sensitivity, but it can also exhibit cross-reactivity among flaviviruses due to their shared antigenic similarities (Calisher et al., 1989). This means that antibodies raised against one flavivirus may react to antigens of other flaviviruses in the ELISA, leading to a false-positive result. Such cross-reactivity is a well-documented issue in flavivirus serology and can result in false-positive ELISA results when testing for a specific flavivirus like TBEV (Chan et al., 2022; Krzysiak et al., 2021; Lim et al., 2018; Marvik et al., 2021; Paulsen et al., 2020). The SNT, on the other hand, is more specific as it directly measures the ability of serum antibodies to neutralize TBEV. If a sample tests negative in SNT but positive in ELISA, it suggests that while there are flavivirus-reactive antibodies present detected by ELISA, these antibodies may not be specific to TBEV (Klaus et al., 2014; Stiasny et al., 2021). This suggests exposure to other flaviviruses closely related to TBE (Stegmüller et al., 2023; Stiasny et al., 2021; Tonteri et al., 2016).

Whole genome sequencing would be valuable for monitoring the spread and adaptation of TBEV variants in Norway (MSIS, 2023). We successfully developed a WGS method at the Norwegian Institute of Public Health for samples with higher viral load (Paper IV). However, field collected samples typically have low viral loads, and enhanced sensitivity and accuracy of our method for detecting viruses at lower concentrations are needed (Andreassen et al., 2012; Vikse et al., 2020).

One issue here is RNA extraction. While we could use relatively gentle, standardises commercially available methods for extraction of RNA from cell culture supernatants, efficient RNA extraction from tick involves mechanical disruption; in addition, it is necessary to freeze the tick. Both these treatments tend to fragment the RNA. Based on decades of experience it has been observed that freshly extracted TBEV RNA are more likely to yield positive PCR results (Personal communication with Professor Åshild K Andreassen).

In our study, primers generating long overlapping fragments were used for WGS to provide thorough genome coverage of cultivated high viral load samples. A limitation in this study was that we were not able to sequence low viral load samples. For such samples, shorter amplicons or combine overlapping fragments of various amplicon lengths would be advantageous (Barandika et al., 2006; Quick et al., 2017; Shi et al., 2021; Zakotnik et al., 2022).

4. Conclusion

This PhD study casts light on TBEV as an emerging virus providing insights on the geographical distribution, prevalence, human incidence, and detection in Scandinavia. Our study indicates a widespread geographical distribution of TBEV in southern Scandinavia that is influenced by climatic conditions. The developed WGS method and bioinformatic analysis provided knowledge of the genetic diversity, evolution, and distribution of two TBEV strains, providing a deeper understanding of the pathogen. The developed WGS methodology lays a foundation for TBEV genomic research in Norway.

5. Future perspectives

Future perspectives for TBEV in Scandinavia include three key points:

Continued surveillance and public awareness

This involves continued surveillance of TBEV in both established and newly emerging tick areas using suitable indicators. The surveillance scope should extend beyond *I. ricinus* and TBEV to encompass other tick species and flaviviruses and capable of TBEV transmission. For example, *I. persulcatus,* is found in northern parts of both Finland and Sweden which can play an important role in the transmission of the TBEV in the region.

Assessment of climate change on TBEV spread

Climate change is expected to alter the distribution of vector borne diseases including TBEV in Scandinavia. Integrating localized microclimate data for TBEV analysis can provide comprehensive understanding of how TBEV transmission is influenced by environmental factors. Future studies should assess the impacts of climate change including relative humidity that could play an important role in TBEV prevalence, more closely and monitor TBE foci within the region, considering the implications for disease transmission.

Focused genetic research and development of improved vaccines

Given the dynamic nature of TBEV and its potential resilience to impacts of climate change, focused genetic research could comprehend knowledge on evolution of TBEV for potential endemic regions. TBEV is prone to adaptation, and this can have implications for genetic variants and higher viral load in tick that could lead to symptomatic infections in future. Advancing the development of vaccines is important, especially considering the genetic variability of TBEV in Scandinavia.

6. References

Achazi, K., Růžek, D., Donoso-Mantke, O., Schlegel, M., Ali, H. S., Wenk, M., Schmidt-Chanasit, J., Ohlmeyer, L., Rühe, F., & Vor, T. (2011). Rodents as sentinels for the prevalence of tick-borne encephalitis virus. *Vector-Borne and Zoonotic Diseases*, *11*(6), 641–647.

Agder fylkeskommune. (2021). *Agder fylkeskommune deltar i utviklingsinitiativ for Jyllandskorridoren*. retrieved from https://agderfk.no/aktuelt/agder-fylkeskommune-deltar-i-utviklingsinitiativ-for-jyllandskorridoren.26614.aspx

Agergaard, C. N., Rosenstierne, M. W., Bødker, R., Rasmussen, M., Andersen, P. H. S., & Fomsgaard, A. (2019). New tick-borne encephalitis virus hot spot in Northern Zealand, Denmark, October 2019. *Eurosurveillance*, *24*(43). https://doi.org/10.2807/1560-7917.ES.2019.24.43.1900639

Alkishe, A. A., Peterson, A. T., & Samy, A. M. (2017). Climate change influences on the potential geographic distribution of the disease vector tick Ixodes ricinus. *PLOS ONE*, *12*(12), e0189092. https://doi.org/10.1371/journal.pone.0189092

Amicizia, D., Domnich, A., Panatto, D., Lai, P. L., Cristina, M. L., Avio, U., & Gasparini, R. (2013). Epidemiology of tick-borne encephalitis (TBE) in Europe and its prevention by available vaccines. *Human Vaccines & Immunotherapeutics*, *9*(5), 1163–1171.

Andersen, N. S., Bestehorn, M., Chitimia-Dobler, L., Kolmos, H. J., Jensen, P. M., Dobler, G., & Skarphédinsson, S. (2019). Phylogenetic characterization of tick-borne encephalitis virus from Bornholm, Denmark. *Ticks and Tick-Borne Diseases*, *10*(3), 533–539. https://doi.org/10.1016/j.ttbdis.2018.12.008

Andersen, N. S., Larsen, S. L., Olesen, C. R., Stiasny, K., Kolmos, H. J., Jensen, P. M., & Skarphédinsson, S. (2019). Continued expansion of tick-borne pathogens: Tick-borne encephalitis virus complex and Anaplasma phagocytophilum in Denmark. *Ticks and Tick-Borne Diseases*, *10*(1), 115–123. https://doi.org/10.1016/j.ttbdis.2018.09.007

Anderson, J. F. (1991). Epizootiology of Lyme borreliosis. *Scandinavian Journal of Infectious Diseases. Supplementum*, *77*, 23–34.

Andreasen, A. M., Dehlendorff, P. B., Knudtzen, F. C., Bødker, R., Kjær, L. J., & Skarphedinsson, S. (2020). Spatial and temporal patterns of Lyme Neuroborreliosis on Funen, Denmark from 1995–2014. *Scientific Reports*, *10*(1), 7796. https://doi.org/10.1038/s41598-020-64638-5

Andreassen, A., Jore, S., Cuber, P., Dudman, S., Tengs, T., Isaksen, K., Hygen, H. O., Viljugrein, H., Ånestad, G., Ottesen, P., & Vainio, K. (2012). Prevalence of tick borne encephalitis virus in tick nymphs in relation to climatic factors on the southern coast of Norway. *Parasites & Vectors*, *5*(1), 177. https://doi.org/10.1186/1756-3305-5-177

Arnell, N. W., & Gosling, S. N. (2013). The impacts of climate change on river flow regimes at the global scale. *Journal of Hydrology*, *486*, 351–364. https://doi.org/10.1016/j.jhydrol.2013.02.010

Asghar, N., Lindblom, P., Melik, W., Lindqvist, R., Haglund, M., Forsberg, P., Överby, A. K., Andreassen, Å., Lindgren, P.-E., & Johansson, M. (2014). Tick-Borne Encephalitis Virus Sequenced Directly from Questing and Blood-Feeding Ticks Reveals Quasispecies Variance. *PLoS ONE*, *9*(7), e103264. https://doi.org/10.1371/journal.pone.0103264

Asghar, N., Pettersson, J. H.-O., Dinnetz, P., Andreassen, Å., & Johansson, M. (2017). Deep sequencing analysis of tick-borne encephalitis virus from questing ticks at natural foci reveals similarities between quasispecies pools of the virus. *Journal of General Virology, 98*(3), 413–421. https://doi.org/10.1099/jgv.0.000704

Bakhvalova, V., Potapova, O., Panov, V., & Morozova, O. (2009). Vertical transmission of tick-borne encephalitis virus between generations of adapted reservoir small rodents. *Virus Research, 140*(1–2), 172–178.

Bandekar, G., & Odland, A. (2017). Ecological characterization of northernmost birch forests in Norway. *Phytocoenologia, 47*(2), 111–124. https://doi.org/10.1127/phyto/2017/0111

Bandekar, G., Vestgarden, L. S., Jenkins, A., & Odland, A. (2020). Bioclimatic gradients and soil property trends from northernmost mainland Norway to the Svalbard archipelago. Does the arctic biome extend into mainland Norway? *PLOS ONE, 15*(9), e0239183. https://doi.org/10.1371/journal.pone.0239183

Barandika, J., Berriatua, E., Barral, M., Juste, R., Anda, P., & García-Pérez, A. (2006). Risk factors associated with ixodid tick species distributions in the Basque region in Spain. *Medical and Veterinary Entomology, 20*(2), 177–188.

Barrows, N. J., Campos, R. K., Liao, K.-C., Prasanth, K. R., Soto-Acosta, R., Yeh, S.-C., Schott-Lerner, G., Pompon, J., Sessions, O. M., & Bradrick, S. S. (2018). Biochemistry and molecular biology of flaviviruses. *Chemical Reviews, 118*(8), 4448–4482.

Belova, O. A., Polienko, A. E., Averianova, A. D., & Karganova, G. G. (2023). Hybrids of Ixodes ricinus and Ixodes persulcatus ticks effectively acquire and transmit tick-borne encephalitis virus. *Frontiers in Cellular and Infection Microbiology, 13*, 1104484. https://doi.org/10.3389/fcimb.2023.1104484

Belozerov, V. N. (2009). Diapause and quiescence as two main kinds of dormancy and their significance in life cycles of mites and ticks (Chelicerata: Arachnida: Acari). *Part, 2*, 3–32.

Benda, R. (1958). The common tick Ixodes ricinus L. as a reservoir and vector of tick-borne encephalitis. I. Survival of the virus (strain B3) during the development of the tick under laboratory conditions. *Journal of Hygiene, Epidemiology, Microbiology and Immunology, 2*.

Bird, T. (2017). *Nordic Action on Climate Change*. Nordic Council of Ministers. https://doi.org/10.6027/ANP2017-766

Borde, J. P., Kaier, K., Hehn, P., Matzarakis, A., Frey, S., Bestehorn, M., Dobler, G., & Chitimia-Dobler, L. (2021). The complex interplay of climate, TBEV vector dynamics and TBEV infection rates in ticks—Monitoring a natural TBEV focus in Germany, 2009–2018. *PLOS ONE*, *16*(1), e0244668. https://doi.org/10.1371/journal.pone.0244668

Box, J. E., Colgan, W. T., Christensen, T. R., Schmidt, N. M., Lund, M., Parmentier, F.-J. W., Brown, R., Bhatt, U. S., Euskirchen, E. S., Romanovsky, V. E., Walsh, J. E., Overland, J. E., Wang, M., Corell, R. W., Meier, W. N., Wouters, B., Mernild, S., Mård, J., Pawlak, J., & Olsen, M. S. (2019). Key indicators of Arctic climate change: 1971–2017. *Environmental Research Letters*, *14*(4), 045010. https://doi.org/10.1088/1748-9326/aafc1b

Bregnard, C., Rais, O., Herrmann, C., Kahl, O., Brugger, K., & Voordouw, M. J. (2021). Beech tree masting explains the inter-annual variation in the fall and spring peaks of Ixodes ricinus ticks with different time lags. *Parasites & Vectors*, *14*(1), 570. https://doi.org/10.1186/s13071-021-05076-8

Brinkley, C., Nolskog, P., Golovljova, I., Lundkvist, Å., & Bergström, T. (2008). Tick-borne encephalitis virus natural foci emerge in western Sweden. *International Journal of Medical Microbiology*, *298*, 73–80. https://doi.org/10.1016/j.ijmm.2007.12.005

Bryn, A. (2008). Recent forest limit changes in south-east Norway: Effects of climate change or regrowth after abandoned utilisation? *Norsk Geografisk Tidsskrift - Norwegian Journal of Geography*, *62*(4), 251–270. https://doi.org/10.1080/00291950802517551

Burri, C., Bastic, V., Maeder, G., Patalas, E., & Gern, L. (2011). Microclimate and the Zoonotic Cycle of Tick-Borne Encephalitis Virus in Switzerland. *Journal of Medical Entomology, 48*(3), 615–627. https://doi.org/10.1603/ME10180

Calisher, C. H., Karabatsos, N., Dalrymple, J. M., Shope, R. E., Porterfield, J. S., Westaway, E. G., & Brandt, W. E. (1989). Antigenic Relationships between Flaviviruses as Determined by Cross-neutralization Tests with Polyclonal Antisera. In *Journal of General Virology* (Vol. 70, Issue 1, pp. 37–43). Microbiology Society. https://doi.org/10.1099/0022-1317-70-1-37

Carpi, G., Cagnacci, F., Neteler, M., & Rizzoli, A. (2008). Tick infestation on roe deer in relation to geographic and remotely sensed climatic variables in a tick-borne encephalitis endemic area. *Epidemiology and Infection*, *136*(10), 1416–1424. https://doi.org/10.1017/S0950268807000039

Carr, A. L., & Salgado, V. L. (2019). Ticks home in on body heat: A new understanding of Haller's organ and repellent action. *PLOS ONE*, *14*(8), e0221659. https://doi.org/10.1371/journal.pone.0221659

Cayol, C., Koskela, E., Mappes, T., Siukkola, A., & Kallio, E. R. (2017). Temporal dynamics of the tick Ixodes ricinus in northern Europe: Epidemiological implications. *Parasites & Vectors*, *10*(1), 166. https://doi.org/10.1186/s13071-017-2112-x

Chamberlain, C. J., & Wolkovich, E. M. (2023). Variation across space, species and methods in models of spring phenology. *Climate Change Ecology*, *5,* 100071. https://doi.org/10.1016/j.ecochg.2023.100071

Chan, K. R., Ismail, A. A., Thergarajan, G., Raju, C. S., Yam, H. C., Rishya, M., & Sekaran, S. D. (2022). Serological cross-reactivity among common flaviviruses. *Frontiers in Cellular and Infection Microbiology, 12,* 975398. https://doi.org/10.3389/fcimb.2022.975398

Chitimia-Dobler, L., Mackenstedt, U., & Petney, T. (2020). Transmission/natural cycle. *The TBE Book, 3rd Ed.; Dobler, G., Erber, W., Schmitt, H.-J., Eds,* 47–63.

Christensen, O. B., Kjellström, E., Dieterich, C., Gröger, M., & Meier, H. E. M. (2022). Atmospheric regional climate projections for the Baltic Sea region until 2100. *Earth System Dynamics*, *13*(1), 133–157. https://doi.org/10.5194/esd-13-133-2022

Crespin, L., Verhagen, R., Stenseth, N. Chr., Yoccoz, N. G., Prévot-Julliard, A.-C., & Lebreton, J.-D. (2002). Survival in fluctuating bank vole populations: Seasonal and yearly variations. *Oikos*, *98*(3), 467–479. https://doi.org/10.1034/j.1600-0706.2002.980311.x

Cuber, P., Dudman, S., Tengs, T., Isaksen, K., Hygen, H. O., Viljugrein, H., Ånestad, G., Ottesen, P., & Vainio, K. (2012). Prevalence of tick borne encephalitis virus in tick nymphs in relation to climatic factors on the southern coast of Norway. *Parasites & Vectors*, *5*(1), 177. https://doi.org/10.1186/1756-3305-5-177

Dahui, Q. (2019). Next-generation sequencing and its clinical application. *Cancer Biology & Medicine, 16*(1), 4–10. https://doi.org/10.20892/j.issn.2095-3941.2018.0055

Dai, X., Shang, G., Lu, S., Yang, J., & Xu, J. (2018). A new subtype of eastern tick-borne encephalitis virus discovered in Qinghai-Tibet Plateau, China. *Emerging Microbes & Infections*, *7*(1), 1–9. https://doi.org/10.1038/s41426-018-0081-6

Daniel, M., Danielová, V., Fialová, A., Malý, M., Kříž, B., & Nuttall, P. A. (2018). Increased Relative Risk of Tick-Borne Encephalitis in Warmer Weather. *Frontiers in Cellular and Infection Microbiology*, *8*, 90. https://doi.org/10.3389/fcimb.2018.00090

Daniel, M., Danielová, V., Kříž, B., Jirsa, A., & Nožička, J. (2003). Shift of the Tick Ixodes ricinus and Tick-Borne Encephalitis to Higher Altitudes in Central Europe. *European Journal of Clinical Microbiology and Infectious Diseases*, *22*(5), 327–328. https://doi.org/10.1007/s10096-003-0918-2

Danielová, V. (1990). Experimental infection of ticks Ixodes ricinus with Tick-borne encephalitis virus under different microclimatic conditions. *Folia Parasitologica*, *37*, 279–282.

Danielová, V., Daniel, M., Holubova, J., Hajkova, Z., Albrecht, V., Marhoul, Z., & Simonova, V. (1983). Influence of microclimatic factors on the development and virus infection rate of ticks Ixodes ricinus (L.) under experimental conditions. *Folia Parasitologica (Praha)*, *30*, 153–161.

Danielová, V., & Holubová, J. (1991). Transovarial transmission rate of tick-borne encephalitis virus in Ixodes ricinus ticks. *Modern Acarology*, *2*, 7–10.

Danielova, V., Holubova, J., Pejcoch, M., & Daniel, M. (2002). Potential significance of transovarial transmission in the circulation of tick-borne encephalitis virus. *Folia Parasitologica*, *49*(4), 323–325.

De Pelsmaeker, N., Korslund, L., & Steifetten, Ø. (2021). High-elevational occurrence of two tick species, Ixodes ricinus and I. trianguliceps, at their northern distribution range. *Parasites & Vectors*, *14*(1), 161. https://doi.org/10.1186/s13071-021-04604-w

Demina, T., Dzhioev, Y. P., Verkhozina, M., Kozlova, I., Tkachev, S., Plyusnin, A., Doroshchenko, E., Lisak, O., & Zlobin, V. (2010). Genotyping and characterization of the geographical distribution of tick-borne encephalitis virus variants with a set of molecular probes. *Journal of Medical Virology*, *82*(6), 965–976.

Dizij, A., & Kurtenbach, K. (1995). Clethrionomys glareolus, but not Apodemus flavicollis, acquires resistance to lxodes ricinus L, the main European vector of Borrelia burgdorferi. *Parasite Immunology*, *17*(4), 177–183. https://doi.org/10.1111/j.1365-3024.1995.tb00887.x

Dobler, G., Hufert, F., Pfeffer, M., & Essbauer, S. (2011). Tick-borne encephalitis: From microfocus to human disease. In *Progress in Parasitology* (pp. 323–331). Springer.

Domingo, E. (1997). Rapid Evolution of Viral RNA Genomes12. *The Journal of Nutrition*, *127*(5), 958S-961S. https://doi.org/10.1093/jn/127.5.958S

Easterling, D. R., Meehl, G. A., Parmesan, C., Changnon, S. A., Karl, T. R., & Mearns, L. O. (2000). Climate Extremes: Observations, Modeling, and Impacts. *Science*, *289*(5487), 2068–2074. https://doi.org/10.1126/science.289.5487.2068

Ebert, T. A., Brlansky, R., & Rogers, M. (2010). Reexamining the pooled sampling approach for estimating prevalence of infected insect vectors. *Annals of the Entomological Society of America*, *103*(6), 827–837.

ECDC. (2021). Tick-borne encephalitis. *In: European Center for Disease Control. Annual Epidemiological Report for 2019*.

Ecker, M., Allison, S. L., Meixner, T., & Heinz, F. X. (1999). Sequence analysis and genetic classification of tick-borne encephalitis viruses from Europe and Asia. *Journal of General Virology*, *80*(1), 179–185.

Elväng, A., Melik, W., Bertrand, Y., Lönn, M., & Johansson, M. (2011). Sequencing of a tick-borne encephalitis virus from Ixodes ricinus reveals a thermosensitive RNA switch significant for virus propagation in ectothermic arthropods. *Vector-Borne and Zoonotic Diseases*, *11*(6), 649–658.

Ergunay, K., Tkachev, S., Kozlova, I., & Růžek, D. (2016). A Review of Methods for Detecting Tick-Borne Encephalitis Virus Infection in Tick, Animal, and Human Specimens. *Vector-Borne and Zoonotic Diseases*, *16*(1), 4–12. https://doi.org/10.1089/vbz.2015.1896

Esser, H. J., Foley, J. E., Bongers, F., Herre, E. A., Miller, M. J., Prins, H. H. T., & Jansen, P. A. (2016). Host body size and the diversity of tick assemblages on Neotropical vertebrates. *International Journal for Parasitology: Parasites and Wildlife*, *5*(3), 295–304. https://doi.org/10.1016/j.ijppaw.2016.10.001

Estrada-Peña, A., Gray, J. S., Kahl, O., Lane, R. S., & Nijhof, A. M. (2013). Research on the ecology of ticks and tick-borne pathogens—Methodological principles and caveats. *Frontiers in Cellular and Infection Microbiology*, *3*. https://doi.org/10.3389/fcimb.2013.00029

Estrada-Peña, A., Martinez, J. M., Sanchez Acedo, C., Quilez, J., & Del Cacho, E. (2004). Phenology of the tick, Ixodes ricinus, in its southern distribution range (central Spain). *Medical and Veterinary Entomology*, *18*(4), 387–397. https://doi.org/10.1111/j.0269-283X.2004.00523.x

Fält, J., Lundgren, Å., Alsterlund, R., Carlsson, B., Eliasson, I., Haglund, M., Lundkvist, Å., Vene, S., & Settergren, B. (2006). Tick-borne encephalitis (TBE) in Skåne, southern Sweden: A new TBE endemic region? *Scandinavian Journal of Infectious Diseases*, *38*(9), 800–804. https://doi.org/10.1080/00365540600664068

Field, C. B., Barros, V. R., & Intergovernmental Panel on Climate Change (Eds.). (2014). *Climate change 2014: Impacts, adaptation, and vulnerability: Working Group II contribution to the fifth assessment report of the Intergovernmental Panel on Climate Change*. Cambridge University Press.

Fomsgaard, A. (2020). TBE in Denmark. Chapter 12b. *The TBE Book*, 198.

Fomsgaard, A., Christiansen, C. B., & Bødker, R. (2009). First identification of tick-borne encephalitis in Denmark outside of Bornholm, August 2009. *Eurosurveillance*, *14*(36). https://doi.org/10.2807/ese.14.36.19325-en

Fomsgaard, A., Fertner, M. E., Essbauer, S., Nielsen, A. Y., Frey, S., Lindblom, P., Lindgren, P.-E., Bødker, R., Weidmann, M., & Dobler, G. (2013). Tick-borne encephalitis virus, Zealand, Denmark, 2011. *Emerging Infectious Diseases*, *19*(7), 1171.

Gaëlle Gonzalez, Laure Bournez, Rayane Amaral Moraes, Dumarest Marine, Clémence Galon, Fabien Vorimore, Maxime Cochin, Antoine Nougairède, Catherine Hennechart-Collette, Sylvie Perelle, Isabelle Leparc-Goffart, Guillaume André Durand, Gilda Grard, Thomas Bénet, Nathalie Danjou, Martine Blanchin, Sandrine A. Lacour, Boué Franck, Guillaume Chenut, … Sylvie Lecollinet. (2022). A One-Health Approach to Investigating an Outbreak of Alimentary Tick-Borne Encephalitis in a Non-endemic Area in France (Ain, Eastern France): A Longitudinal Serological Study in Livestock, Detection in Ticks, and the First Tick-Borne Encephalitis Virus Isolation and Molecular Characterisation. *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2022.863725

Gäumann, R., Mühlemann, K., Strasser, M., & Beuret, C. M. (2010). High-Throughput Procedure for Tick Surveys of Tick-Borne Encephalitis Virus and Its Application in a National Surveillance Study in Switzerland. *Applied and Environmental Microbiology*, *76*(13), 4241–4249. https://doi.org/10.1128/AEM.00391-10

Gilbert, L., Jones, L., Hudson, P. J., Gould, E., & Reid, H. (2000). Role of small mammals in the persistence of Louping-ill virus: Field survey and tick co-feeding studies. *Medical and Veterinary Entomology*, *14*(3), 277–282.

Girl, P., Haut, M., Riederer, S., Pfeffer, M., & Dobler, G. (2021). Comparison of Three Serological Methods for the Epidemiological Investigation of TBE in Dogs. *Microorganisms*, *9*(2), 399. https://doi.org/10.3390/microorganisms9020399

Gray, J. (1991). The development and seasonal activity of the tick Ixodes ricinus: A vector of Lyme borreliosis. *Review of Medical and Veterinary Entomology*, *79*(6), 323–333.

Gray, J. S., Dautel, H., Estrada-Peña, A., Kahl, O., & Lindgren, E. (2009). Effects of Climate Change on Ticks and Tick-Borne Diseases in Europe. *Interdisciplinary Perspectives on Infectious Diseases*, *2009*, 1–12. https://doi.org/10.1155/2009/593232

Gray, J. S., Kahl, O., Lane, R. S., Levin, M. L., & Tsao, J. I. (2016). Diapause in ticks of the medically important Ixodes ricinus species complex. *Ticks and Tick-Borne Diseases, 7*(5), 992–1003.

Gregory, R. D., Willis, S. G., Jiguet, F., Voříšek, P., Klvaňová, A., van Strien, A., Huntley, B., Collingham, Y. C., Couvet, D., & Green, R. E. (2009). An indicator of the impact of climatic change on European bird populations. *PloS One*, *4*(3), e4678.

Gritsun, T. S., Frolova, T. V., Zhankov, A. I., Armesto, M., Turner, S. L., Frolova, M. P., Pogodina, V. V., Lashkevich, V. A., & Gould, E. A. (2003). Characterization of a Siberian Virus Isolated from a Patient with Progressive Chronic Tick-Borne Encephalitis. *Journal of Virology*, *77*(1), 25–36. https://doi.org/10.1128/JVI.77.1.25-36.2003

Grubaugh, N. D., Gangavarapu, K., Quick, J., Matteson, N. L., De Jesus, J. G., Main, B. J., Tan, A. L., Paul, L. M., Brackney, D. E., Grewal, S., Gurfield, N., Van Rompay, K. K. A., Isern, S., Michael, S. F., Coffey, L. L., Loman, N. J., & Andersen, K. G. (2019). An amplicon-based sequencing framework for accurately measuring intrahost virus diversity using PrimalSeq and iVar. *Genome Biology*, *20*(1), 8. https://doi.org/10.1186/s13059-018-1618-7

Haglund, M., Settergren, B., Heinz, F. X., & Günther, G. (2003). Report of the Meningitis Program of the International Scientific Working Group on TBE: Serological screening of patients with viral CNS-infection of unknown etiology in search of undiagnosed TBE cases. *Vaccine*, *21*, S66–S72. https://doi.org/10.1016/S0264-410X(02)00816-2

Haglund, M., Vene, S., Forsgren, M., Günther, G., Johansson, B., Niedrig, M., Plyusnin, A., Lindquist, L., & Lundkvist, Å. (2003). Characterisation of human tick-borne encephalitis virus from Sweden. *Journal of Medical Virology*, *71*(4), 610–621.

Hallang, H., Froyd, C. A., Hiemstra, J. F., & Los, S. O. (2022). Tree line shifts, changing vegetation assemblages and permafrost dynamics on Galdhøpiggen (Jotunheimen, Norway) over the past ~4400 years. *The Holocene*, *32*(4), 308–320. https://doi.org/10.1177/09596836211066591

Hanssen-Bauer, I., Førland, E., Haddeland, I., Hisdal, H., Lawrence, D., Mayer, S., Nesje, A., Nilsen, J. E., Sandven, S., Sandø, A., Sorteberg, A., & Ådlandsvik, B. (2017). *Climate in Norway 2100*.

Hasle, G., Bjune, G., Edvardsen, E., Jakobsen, C., Linnehol, B., Røer, J. E., Mehl, R., Røed, K. H., Pedersen, J., & Leinaas, H. P. (2009). Transport of ticks by migratory passerine birds to Norway. *Journal of Parasitology, 95*(6), 1342–1351.

Hauser, G., Rais, O., Morán Cadenas, F., Gonseth, Y., Bouzelboudjen, M., & Gern, L. (2018). Influence of climatic factors on Ixodes ricinus nymph abundance and phenology over a long-term monthly observation in Switzerland (2000–2014). *Parasites & Vectors*, *11*(1), 1–12.

Havlíková, S., Ličková, M., & Klempa, B. (2013). Non-viraemic transmission of tick-borne viruses. *Acta Virologica*, *57*(02), 123–129. https://doi.org/10.4149/av_2013_02_123

Hedenås, H., Christensen, P., & Svensson, J. (2016). Changes in vegetation cover and composition in the Swedish mountain region. *Environmental Monitoring and Assessment*, *188*(8), 452. https://doi.org/10.1007/s10661-016-5457-2

Heinze, D. M., Gould, E. A., & Forrester, N. L. (2012). Revisiting the Clinal Concept of Evolution and Dispersal for the Tick-Borne Flaviviruses by Using Phylogenetic and

Biogeographic Analyses. *Journal of Virology*, *86*(16), 8663–8671. https://doi.org/10.1128/JVI.01013-12

Helmová, R., Hönig, V., Tykalová, H., Palus, M., Bell-Sakyi, L., & Grubhoffer, L. (2020). Tick-Borne Encephalitis Virus Adaptation in Different Host Environments and Existence of Quasispecies. *Viruses*, *12*(8), 902. https://doi.org/10.3390/v12080902

Henningsson, A. J., Lindqvist, R., Norberg, P., Lindblom, P., Roth, A., Forsberg, P., Bergström, T., Överby, A. K., & Lindgren, P.-E. (2016). Human Tick-Borne Encephalitis and Characterization of Virus from Biting Tick. *Emerging Infectious Diseases*, *22*(8), 1485–1487. https://doi.org/10.3201/eid2208.151962

Heyman, P., Cochez, C., Hofhuis, A., Van Der Giessen, J., Sprong, H., Porter, S. R., Losson, B., Saegerman, C., Donoso-Mantke, O., & Niedrig, M. (2010). A clear and present danger: Tick-borne diseases in Europe. *Expert Review of Anti-Infective Therapy*, *8*(1), 33–50.

Hillyard, P. D. (1996). *Ticks of north-west Europe*. Field Studies Council.

Holmgren, E. B., & Forsgren, M. (1990). Epidemiology of Tick-borne Encephalitis in Sweden 1956–1989: A Study of 1116 Cases. *Scandinavian Journal of Infectious Diseases*, *22*(3), 287–295. https://doi.org/10.3109/00365549009027050

Holzmann, H., Aberle, S. W., Stiasny, K., Werner, P., Mischak, A., Zainer, B., Netzer, M., Koppi, S., Bechter, E., & Heinz, F. X. (2009). Tick-borne Encephalitis from Eating Goat Cheese in a Mountain Region of Austria. *Emerging Infectious Diseases*, *15*(10), 1671–1673. https://doi.org/10.3201/eid1510.090743

Hurrell, J. W., & Van Loon, H. (1997). Decadal Variations in Climate Associated with the North Atlantic Oscillation. In H. F. Diaz, M. Beniston, & R. S. Bradley (Eds.), *Climatic Change at High Elevation Sites* (pp. 69–94). Springer Netherlands. https://doi.org/10.1007/978-94-015-8905-5_4

Hvidsten, D., Frafjord, K., Gray, J. S., Henningsson, A. J., Jenkins, A., Kristiansen, B. E., Lager, M., Rognerud, B., Slåtsve, A. M., & Stordal, F. (2020). The distribution limit of the common tick, Ixodes ricinus, and some associated pathogens in north-western Europe. *Ticks and Tick-Borne Diseases*, *11*(4), 101388.

Imhoff, M., Hagedorn, P., Schulze, Y., Hellenbrand, W., Pfeffer, M., & Niedrig, M. (2015). Review: Sentinels of tick-borne encephalitis risk. *Ticks and Tick-Borne Diseases*, *6*(5), 592–600. https://doi.org/10.1016/j.ttbdis.2015.05.001

Jaaskelainen, A. E., Sironen, T., Murueva, G. B., Subbotina, N., Alekseev, A. N., Castren, J., Alitalo, I., Vaheri, A., & Vapalahti, O. (2010). Tick-borne encephalitis virus in ticks in Finland, Russian Karelia and Buryatia. *Journal of General Virology*, *91*(11), 2706–2712. https://doi.org/10.1099/vir.0.023663-0

Jaenson, T. G., Erik H. Petersson, Petersson, E., Jaenson, D. G. E., Kindberg, J., Pettersson, J. H.-O., Hjertqvist, M., Medlock, J. M., & Bengtsson, H. (2018). The importance of wildlife in the ecology and epidemiology of the TBE virus in Sweden: Incidence of human TBE correlates with abundance of deer and hares. *Parasites & Vectors*, *11*(1), 477–477. https://doi.org/10.1186/s13071-018-3057-4

Jaenson, T. G. T., Jaenson, D. G., Eisen, L., Petersson, E., & Lindgren, E. (2012). Changes in the geographical distribution and abundance of the tick Ixodes ricinus during the past 30 years in Sweden. *Parasites & Vectors*, *5*(1), 8. https://doi.org/10.1186/1756-3305-5-8

Jaenson, T. G. T., & Lindgren, E. (2011). The range of Ixodes ricinus and the risk of contracting Lyme borreliosis will increase northwards when the vegetation period becomes longer. *Ticks and Tick-Borne Diseases*, *2*(1), 44–49. https://doi.org/10.1016/j.ttbdis.2010.10.006

Jaenson, T. G. T., & Wilhelmsson, P. (2019). First records of tick-borne pathogens in populations of the taiga tick Ixodes persulcatus in Sweden. *Parasites & Vectors*, *12*(1), 559. https://doi.org/10.1186/s13071-019-3813-0

Jore, S., Vanwambeke, S. O., Viljugrein, H., Isaksen, K., Kristoffersen, A. B., Woldehiwet, Z., Johansen, B., Brun, E., Brun-Hansen, H., Westermann, S., Larsen, I.-L., Ytrehus, B., & Hofshagen, M. (2014). Climate and environmental change drives Ixodes ricinus geographical expansion at the northern range margin. *Parasites & Vectors*, *7*(1), 11. https://doi.org/10.1186/1756-3305-7-11

Jore, S., Viljugrein, H., Hofshagen, M., Brun-Hansen, H., Kristoffersen, A. B., Nygård, K., Brun, E., Ottesen, P., Sævik, B. K., & Ytrehus, B. (2011). Multi-source analysis reveals latitudinal and altitudinal shifts in range of Ixodes ricinus at its northern distribution limit. *Parasites & Vectors*, *4*(1), 84–84. https://doi.org/10.1186/1756-3305-4-84

Kahl, O. (1991). *Investigations on the water balance of ticks (Acari: Ixodoidea) in the course of their postembryonic development with special reference to active water vapour uptake of the engorged phases.*

Kahl, O., & Gray, J. S. (2023). The biology of Ixodes ricinus with emphasis on its ecology. *Ticks and Tick-Borne Diseases*, *14*(2), 102114. https://doi.org/10.1016/j.ttbdis.2022.102114

Kahl, O., Kämmer, D., & Heger, J. (n.d.). *Duration of the life cycle of Ixodes ricinus in Central Europe and its seasonality–old beliefs revisited. 28*.

Kahl, O., Pogodina, V. V., Poponnikova, T., Süss, J., & Zlobin, V. I. (2023). Chapter 1: A short history of TBE. *Tick-Borne Encephalitis - The Book*. https://doi.org/10.33442/26613980_1-6

Kaiser, R. (2002). Tick-borne encephalitis (TBE) in Germany and clinical course of the disease. *International Journal of Medical Microbiology*, *291*, 58–61. https://doi.org/10.1016/S1438-4221(02)80012-1

Kiffner, C., Vor, T., Hagedorn, P., Niedrig, M., & Rühe, F. (2012). Determinants of tick-borne encephalitis virus antibody presence in roe deer (Capreolus capreolus) sera. *Medical and Veterinary Entomology*, *26*(1), 18–25. https://doi.org/10.1111/j.1365-2915.2011.00961.x

Kjær, L. J., Johansson, M., Lindgren, P.-E., Asghar, N., Wilhelmsson, P., Fredlund, H., Christensson, M., Wallenhammar, A., Bødker, R., Rasmussen, G., & Kjellander, P. (2023). Potential drivers of human tick-borne encephalitis in the Örebro region of Sweden, 2010–2021. *Scientific Reports*, *13*(1), 7685. https://doi.org/10.1038/s41598-023-34675-x

Kjær, L. J., Soleng, A., Edgar, K. S., Lindstedt, H. E. H., Paulsen, K. M., Andreassen, Å. K., Korslund, L., Kjelland, V., Slettan, A., Stuen, S., Kjellander, P., Christensson, M., Teräväinen, M., Baum, A., Klitgaard, K., & Bødker, R. (2019). Predicting and mapping human risk of exposure to Ixodes ricinus nymphs using climatic and environmental data, Denmark, Norway and Sweden, 2016. *Eurosurveillance*, *24*(9). https://doi.org/10.2807/1560-7917.ES.2019.24.9.1800101

Klaus, C., Beer, M., Saier, R., Schau, U., Moog, U., Hoffmann, B., Diller, R., & Süss, J. (2012). Goats and sheep as sentinels for tick-borne encephalitis (TBE) virus–epidemiological studies in areas endemic and non-endemic for TBE virus in Germany. *Ticks and Tick-Borne Diseases*, *3*(1), 27–37.

Klaus, C., Gethmann, J., Hoffmann, B., Ziegler, U., Heller, M., & Beer, M. (2016). Tick infestation in birds and prevalence of pathogens in ticks collected from different places in Germany. *Parasitology Research*, *115*(7), 2729–2740. https://doi.org/10.1007/s00436-016-5022-5

Klaus, C., Ziegler, U., Kalthoff, D., Hoffmann, B., Hoffmann, B., & Beer, M. (2014). Tick-borne encephalitis virus (TBEV) – findings on cross reactivity and longevity of TBEV antibodies in animal sera. *BMC Veterinary Research*, *10*(1), 78–78. https://doi.org/10.1186/1746-6148-10-78

Kofler, R. M., Heinz, F. X., & Mandl, C. W. (2002). Capsid protein C of tick-borne encephalitis virus tolerates large internal deletions and is a favorable target for attenuation of virulence. *Journal of Virology*, *76*(7), 3534–3543. https://doi.org/10.1128/jvi.76.7.3534-3543.2002

Korenberg, E. (2009). Recent epidemiology of tick-borne encephalitis: An effect of climate change? *Advances in Virus Research*, *74*, 123–144.

Korenberg, E. I., Sirotkin, M. B., & Kovalevskii, Yu. V. (2021). Adaptive Features of the Biology of Closely Related Species of Ixodid Ticks That Determine Their Distribution (Illustrated on the Example of the Taiga Tick Ixodes persulcatus Sch. 1930 and the Castor Bean Tick Ixodes ricinus L. 1758). *Biology Bulletin Reviews*, *11*(6), 602–615. https://doi.org/10.1134/S2079086421060050

Kovalev, S., & Mukhacheva, T. (2017). Reconsidering the classification of tick-borne encephalitis virus within the Siberian subtype gives new insights into its evolutionary history. *Infection, Genetics and Evolution*, *55*, 159–165.

Kovalev, S. Y., Golovljova, I. V., & Mukhacheva, T. A. (2016). Natural hybridization between Ixodes ricinus and Ixodes persulcatus ticks evidenced by molecular genetics methods. *Ticks and Tick-Borne Diseases*, *7*(1), 113–118. https://doi.org/10.1016/j.ttbdis.2015.09.005

Kozlova, I. V., Verkhozina, M. M., Demina, T. V., Dzhioev, Yu. P., Tkachev, S. E., Karan, L. S., Doroshchenko, E. K., Lisak, O. V., Suntsova, O. V., Paramonov, A. I., Fedulina, O. O., Revizor, A. O., & Zlobi, V. I. (2013). Genetic and Biological Properties of Original TBEV Strains Group Circulating in Eastern Siberia. In S. Tkachev (Ed.), *Encephalitis*. InTech. https://doi.org/10.5772/54087

Kristiansen, K. (2002). TBE in Denmark-in particular on Bornholm. *International Journal of Medical Microbiology*, *291*, 62.

Krzysiak, M. K., Anusz, K., Konieczny, A., Rola, J., Salat, J., Strakova, P., Olech, W., & Larska, M. (2021). The European bison (Bison bonasus) as an indicatory species for the circulation of tick-borne encephalitis virus (TBEV) in natural foci in Poland. *Ticks and Tick-Borne Diseases*, *12*(6), 101799. https://doi.org/10.1016/j.ttbdis.2021.101799

Kullman, L. (1997). A 25-year survey of geoecological change in the scandes mountains of sweden. *Geografiska Annaler: Series A, Physical Geography*, *79*(3), 139–165. https://doi.org/10.1111/j.0435-3676.1997.00012.x

Kullman, L. (2008). Thermophilic tree species reinvade subalpine Sweden–early responses to anomalous late Holocene climate warming. *Arctic, Antarctic, and Alpine Research*, *40*(1), 104–110.

Kullman, L., & Öberg, L. (2022). Recent and Past Arboreal Change: Observational and Retrospective Studies Within a Subalpine, Birch-Dominated (Betula Pubescens Ssp. Czerepanovii) Mountain Valley in the Southern Swedish Scandes-Responses to Climate Change and Land Use. *European Journal of Applied Sciences*, *10*(6). https://doi.org/10.14738/aivp.106.13467

Kunze, M., Banović, P., Bogovič, P., Briciu, V., Čivljak, R., Dobler, G., Hristea, A., Kerlik, J., Kuivanen, S., Kynčl, J., Lebech, A.-M., Lindquist, L., Paradowska-Stankiewicz, I., Roglić, S., Smíšková, D., Strle, F., Vapalahti, O., Vranješ, N., Vynograd, N., … Erber,

W. (2022). Recommendations to Improve Tick-Borne Encephalitis Surveillance and Vaccine Uptake in Europe. *Microorganisms, 10*(7), 1283. https://doi.org/10.3390/microorganisms10071283

Kurtenbach, K., Kampen, H., Dizij, A., Arndt, S., Seitz, H. M., Schaible, U. E., & Simon, M. M. (1995). Infestation of Rodents with Larval Ixodes ricinus (Acari; Ixodidae) Is an Important Factor in the Transmission Cycle of Borrelia burgdorferi s.l. In German Woodlands. *Journal of Medical Entomology*, *32*(6), 807–817. https://doi.org/10.1093/jmedent/32.6.807

Kutschera, L. S., & Wolfinger, M. T. (2022). Evolutionary traits of Tick-borne encephalitis virus: Pervasive non-coding RNA structure conservation and molecular epidemiology. *Virus Evolution*, *8*(1), veac051. https://doi.org/10.1093/ve/veac051

Kvapil, P., Račnik, J., Kastelic, M., Pittermannová, P., Avšič-Zupanc, T., Bártová, E., & Sedlák, K. (2021). Detection of Antibodies Against Tick-Borne Encephalitis Virus and Other Flaviviruses in a Zoological Collection in Slovenia. *Frontiers in Veterinary Science*, *8*, 688904. https://doi.org/10.3389/fvets.2021.688904

Labuda, M., Jones, L. D., Williams, T., Danielova, V., & Nuttall, P. A. (1993). Efficient transmission of tick-borne encephalitis virus between cofeeding ticks. *Journal of Medical Entomology, 30*(1), 295–299.

Lamsal, A., Tryland, M., Paulsen, K. M., Romano, J. S., Nymo, I. H., Stiasny, K., Soleng, A., Vikse, R., & Andreassen, Å. K. (2023). Serological screening for tick-borne encephalitis virus in eight Norwegian herds of semi-domesticated reindeer (*RANGIFER TARANDUS TARANDUS*). *Zoonoses and Public Health*, zph.13060. https://doi.org/10.1111/zph.13060

Langvall, O., & Ottosson Löfvenius, M. (2021). Long-term standardized forest phenology in Sweden: A climate change indicator. *International Journal of Biometeorology*, *65*(3), 381–391. https://doi.org/10.1007/s00484-019-01817-8

Larsen, A. L., Kanestrøm, A., Bjørland, M., Andreassen, Å., Soleng, A., Vene, S., & Dudman, S. G. (2014). Detection of specific IgG antibodies in blood donors and tick-borne encephalitis virus in ticks within a non-endemic area in southeast Norway. *Scandinavian Journal of Infectious Diseases*, *46*(3), 181–184. https://doi.org/10.3109/00365548.2013.865140

Lejal, E., Moutailler, S., Šimo, L., Vayssier-Taussat, M., & Pollet, T. (2019). Tick-borne pathogen detection in midgut and salivary glands of adult Ixodes ricinus. *Parasites & Vectors*, *12*(1), 1–8.

Ličková, M., Fumačová Havlíková, S., Sláviková, M., & Klempa, B. (2021). Alimentary Infections by Tick-Borne Encephalitis Virus. *Viruses*, *14*(1), 56. https://doi.org/10.3390/v14010056

Liebig, K., Boelke, M., Grund, D., Schicht, S., Bestehorn-Willmann, M., Chitimia-Dobler, L., Dobler, G., Jung, K., & Becker, S. C. (2021). The Stable Matching Problem in TBEV Enzootic Circulation: How Important Is the Perfect Tick-Virus Match? *Microorganisms*, *9*(1), 196. https://doi.org/10.3390/microorganisms9010196

Liebig, K., Boelke, M., Grund, D., Schicht, S., Springer, A., Strube, C., Chitimia-Dobler, L., Dobler, G., Jung, K., & Becker, S. (2020). Tick populations from endemic and non-endemic areas in Germany show differential susceptibility to TBEV. *Scientific Reports*, *10*(1), 15478. https://doi.org/10.1038/s41598-020-71920-z

Lim, S. M., Geervliet, M., Verhagen, J. H., Müskens, G. J. D. M., Majoor, F. A., Osterhaus, A. D. M. E., & Martina, B. E. E. (2018). Serologic evidence of West Nile virus and Usutu virus infections in Eurasian coots in the Netherlands. *Zoonoses and Public Health*, *65*(1), 96–102. https://doi.org/10.1111/zph.12375

Lind, P., & Kjellström, E. (2008). *Temperature and precipitation changes in Sweden; a wide range of model-based projections for the 21st century*. SMHI.

Lindblom, P., Wilhelmsson, P., Fryland, L., Sjöwall, J., Haglund, M., Matussek, A., Ernerudh, J., Vene, S., Nyman, D., Andreassen, Å., Forsberg, P., & Lindgren, P.-E. (2014). Tick-borne encephalitis virus in ticks detached from humans and follow-up of serological and clinical response. *Ticks and Tick-Borne Diseases*, *5*(1), 21–28. https://doi.org/10.1016/j.ttbdis.2013.07.009

Lindgren, E., Andersson, Y., Suk, J. E., Sudre, B., & Semenza, J. C. (2012). Monitoring EU Emerging Infectious Disease Risk Due to Climate Change. *Science*, *336*(6080), 418–419. https://doi.org/10.1126/science.1215735

Lindgren, E., Tälleklint, L., & Polfeldt, T. (2000). Impact of climatic change on the northern latitude limit and population density of the disease-transmitting European tick Ixodes ricinus. *Environmental Health Perspectives, 108*(2), 119–123.

Lindquist, L., & Vapalahti, O. (2008). Tick-borne encephalitis. *The Lancet*, *371*(9627), 1861–1871. https://doi.org/10.1016/S0140-6736(08)60800-4

Lindqvist, R., Kurhade, C., Gilthorpe, J. D., & Överby, A. K. (2018). Cell-type- and region-specific restriction of neurotropic flavivirus infection by viperin. *Journal of Neuroinflammation*, *15*(1), 80. https://doi.org/10.1186/s12974-018-1119-3

LitzbaNadine, ZelenáHana, KreilThomas, R., NiklassonBo, Kühlmann-RabensIlona, Elena, R., & NiedrigMatthias. (2014). Evaluation of Different Serological Diagnostic Methods for Tick-Borne Encephalitis Virus: Enzyme-Linked Immunosorbent, Immunofluorescence, and Neutralization Assay. *Vector-Borne and Zoonotic Diseases*. https://doi.org/10.1089/vbz.2012.1287

Løkken, J. O., Evju, M., Söderström, L., & Hofgaard, A. (2020). Vegetation response to climate warming across the forest–tundra ecotone: Species-dependent upward movement. *Journal of Vegetation Science*, *31*(5), 854–866. https://doi.org/10.1111/jvs.12911

Lukan, M., Bullova, E., & Petko, B. (2010). Climate warming and tick-borne encephalitis, Slovakia. *Emerging Infectious Diseases*, *16*(3), 524.

Lundkvist, Å., Wallensten, A., Vene, S., & Hjertqvist, M. (2011). Tick-borne encephalitis increasing in Sweden, 2011. *Eurosurveillance*, *16*(39), 19981.

Madder, M., Horak, I., & Stoltsz, H. (2014). Tick identification. *Pretoria: Faculty of Veterinary Science University of Pretoria*, *58*.

Marvik, Å., Tveten, Y., Pedersen, A.-B., Stiasny, K., Andreassen, Å. K., & Grude, N. (2021). Low prevalence of tick-borne encephalitis virus antibodies in Norwegian blood donors. *Infectious Diseases*, *53*(1), 44–51. https://doi.org/10.1080/23744235.2020.1819561

Materna, J., Daniel, M., & Danielová, V. (2005). Altitudinal distribution limit of the tick Ixodes ricinus shifted considerably towards higher altitudes in central Europe: Results of three years monitoring in the Krkonose Mts.(Czech Republic). *Central European Journal of Public Health*, *13*(1), 24–28.

Mehl, R. (1983). *The distribution and host relations of Norwegian ticks (Acari, Ixodides)*.

Melik, W., Nilsson, A. S., & Johansson, M. (2007). Detection strategies of tick-borne encephalitis virus in Swedish Ixodes ricinus reveal evolutionary characteristics of emerging tick-borne flaviviruses. *Archives of Virology*, *152*(5), 1027–1034. https://doi.org/10.1007/s00705-006-0922-9

Menzel, A., Sparks, T. H., Estrella, N., Koch, E., Aasa, A., Ahas, R., Alm-Kübler, K., Bissolli, P., Braslavská, O., Briede, A., Chmielewski, F. M., Crepinsek, Z., Curnel, Y., Dahl, Å., Defila, C., Donnelly, A., Filella, Y., Jatczak, K., Måge, F., … Zust, A. (2006). European phenological response to climate change matches the warming pattern: EUROPEAN

PHENOLOGICAL RESPONSE TO CLIMATE CHANGE. *Global Change Biology*, *12*(10), 1969–1976. https://doi.org/10.1111/j.1365-2486.2006.01193.x

Michelitsch, A., Wernike, K., Klaus, C., Dobler, G., & Beer, M. (2019). Exploring the Reservoir Hosts of Tick-Borne Encephalitis Virus. *Viruses*, *11*(7), 669. https://doi.org/10.3390/v11070669

Moen, J., Aune, K., Edenius, L., & Angerbjörn, A. (2004). Potential Effects of Climate Change on Treeline Position in the Swedish Mountains. *Ecology and Society*, *9*(1), art16. https://doi.org/10.5751/ES-00634-090116

MSIS. (2023). *Tick-borne encephalitis*. Norwegian surveillance system for Communicable diseases. https://www.fhi.no/nettpub/smittevernveilederen/sykdommer-a-a/skogflattencefalitt-tbe-virusinfeks

Mysterud, A. (2000). Diet overlap among ruminants in Fennoscandia. *Oecologia*, *124*, 130–137.

Mysterud, A., Hatlegjerde, I. L., & Sørensen, O. J. (2014). Attachment site selection of life stages of Ixodes ricinus ticks on a main large host in Europe, the red deer (Cervus elaphus). *Parasites & Vectors*, *7*, 1–5.

Mysterud, A., Stigum, V. M., Seland, I. V., Herland, A., Easterday, W. R., Jore, S., Østerås, O., & Viljugrein, H. (2018). Tick abundance, pathogen prevalence, and disease incidence in two contrasting regions at the northern distribution range of Europe. *Parasites & Vectors*, *11*(1), 309. https://doi.org/10.1186/s13071-018-2890-9

Nah, K., Bede-Fazekas, Á., Trájer, A. J., & Wu, J. (2020). The potential impact of climate change on the transmission risk of tick-borne encephalitis in Hungary. *BMC Infectious Diseases*, *20*(1), 34. https://doi.org/10.1186/s12879-019-4734-4

National Veterinary Institute. (2022). *Surveillance of infectious diseases in animals and humans in Sweden* (1654–7098; SVA:S Rapportserie 89). https://www.sva.se/media/wlghzh1z/tick-borne-encephalitis.pdf

Norden. (2009). *Signs of Climate Change in Nordic Nature*. Nordic Council of Ministers.

Nuttall, P., & Labuda, M. (2003). Dynamics of infection in tick vectors and at the tick-host interface. *Adv Virus Res*, *60*, 233–272.

Ogden, N. H., Ben Beard, C., Ginsberg, H. S., & Tsao, J. I. (2021). Possible Effects of Climate Change on Ixodid Ticks and the Pathogens They Transmit: Predictions and Observations. *Journal of Medical Entomology*, *58*(4), 1536–1545. https://doi.org/10.1093/jme/tjaa220

Omazic, A., Han, S., Albihn, A., Ullman, K., Choklikitumnuey, P., Perissinotto, D., & Grandi, G. (2023). Ixodid tick species found in northern Sweden – Data from a frontier area. *Ticks and Tick-Borne Diseases*, *14*(6), 102244. https://doi.org/10.1016/j.ttbdis.2023.102244

Omazic, A., Wallenhammar, A., Lahti, E., Asghar, N., Hanberger, A., Hjertqvist, M., Johansson, M., & Albihn, A. (2023). Dairy milk from cow and goat as a sentinel for tick-borne encephalitis virus surveillance. *Comparative Immunology, Microbiology and Infectious Diseases*, *95*, 101958. https://doi.org/10.1016/j.cimid.2023.101958

Patel, P., Landt, O., Kaiser, M., Faye, O., Koppe, T., Lass, U., Sall, A. A., & Niedrig, M. (2013). Development of one-step quantitative reverse transcription PCR for the rapid detection of flaviviruses. *Virology Journal*, *10*(1), 58. https://doi.org/10.1186/1743-422X-10-58

Paulsen, K. M., Lamsal, A., Bastakoti, S., Pettersson, J. H.-O., Pedersen, B. N., Stiasny, K., Haglund, M., Smura, T., Vapalahti, O., Vikse, R., Alfsnes, K., & Andreassen, Å. K. (2021). High-throughput sequencing of two European strains of tick-borne encephalitis virus (TBEV), Hochosterwitz and 1993/783. *Ticks and Tick-Borne Diseases*, *12*(1), 101557. https://doi.org/10.1016/j.ttbdis.2020.101557

Paulsen, K. M., Neves, C. G. das, Granquist, E. G., Madslien, K., Stuen, S., Pedersen, B. N., Vikse, R., Rocchi, M., Mara Rocchi, Ellie Laming, Laming, E., Stiasny, K., & Andreassen, Å. K. (2020). Cervids as sentinel-species for tick-borne encephalitis virus in Norway—A serological study. *Zoonoses and Public Health*, *67*(4), 342–351. https://doi.org/10.1111/zph.12675

Paulsen, K. M., Pedersen, B. N., Benedikte N. Pedersen, Soleng, A., Okbaldet, Y. B., Pettersson, J. H.-O., Dudman, S. G., Ottesen, P., Vik, I. S. S., Vainio, K., & Andreassen, Å. K. (2015). Prevalence of tick-borne encephalitis virus in Ixodes ricinus ticks from three islands in north-western Norway. *Apmis*, *123*(9), 759–764. https://doi.org/10.1111/apm.12412

Paulsen, K. M., Stuen, S., Neves, C. G. das, Suhel, F., Gurung, D., Soleng, A., Stiasny, K., Vikse, R., Andreassen, Å. K., & Granquist, E. G. (2019). Tick-borne encephalitis virus in cows and unpasteurized cow milk from Norway. *Zoonoses and Public Health*, *66*(2), 216–222. https://doi.org/10.1111/zph.12554

Perez, G., Bastian, S., Chastagner, A., Agoulon, A., Plantard, O., Vourc'h, G., & Butet, A. (2017). Ecological factors influencing small mammal infection by Anaplasma phagocytophilum and Borrelia burgdorferi sl in agricultural and forest landscapes. *Environmental Microbiology*, *19*(10), 4205–4219.

Perret, J.-L., Rais, O., & Gern, L. (2004). Influence of Climate on the Proportion of <I>Ixodes ricinus</I> Nymphs and Adults Questing in a Tick Population. *Journal of Medical Entomology*, *41*(3), 361–365. https://doi.org/10.1603/0022-2585-41.3.361

Pettersson, J.-O., Golovljova, I., Vene, S., & Jaenson, T. G. (2014). Prevalence of tick-borne encephalitis virus in Ixodes ricinus ticks in northern Europe with particular reference to

Southern Sweden. *Parasites & Vectors*, *7*(1), 102. https://doi.org/10.1186/1756-3305-7-102

Public Health Agency of Sweden. (2023a). *Areas with increased incidence of TBE*. https://www.folkhalsomyndigheten.se/smittskydd-beredskap/smittsamma-sjukdomar/tick-borne-encephalitis-tbe/omraden-med-forhojd-forekomst-av-tbe/

Public Health Agency of Sweden. (2023b). *Tick-borne encephalitis (TBE)-disease statistics 2013-2023*. https://www.folkhalsomyndigheten.se/folkhalsorapportering-statistik/statistik-a-o/sjukdomsstatistik/tick-borne-encephalitis-tbe/?t=county

Pucek, Z., Jędrzejewski, W., Jędrzejewska, B., & Pucek, M. (1993). Rodent population dynamics in a primeval deciduous forest (Białowieża National Park) in relation to weather, seed crop, and predation. *Acta Theriologica*, *38*, 199–232. https://doi.org/10.4098/AT.arch.93-18

Pulkkinen, L., Butcher, S., & Anastasina, M. (2018). Tick-Borne Encephalitis Virus: A Structural View. *Viruses*, *10*(7), 350. https://doi.org/10.3390/v10070350

Quick, J., Grubaugh, N. D., Pullan, S. T., Claro, I. M., Smith, A. D., Gangavarapu, K., Oliveira, G., Robles-Sikisaka, R., Rogers, T. F., Beutler, N. A., Burton, D. R., Lewis-Ximenez, L. L., De Jesus, J. G., Giovanetti, M., Hill, S. C., Black, A., Bedford, T., Carroll, M. W., Nunes, M., … Loman, N. J. (2017). Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. *Nature Protocols*, *12*(6), 1261–1276. https://doi.org/10.1038/nprot.2017.066

Qviller, L., Grøva, L., Viljugrein, H., Klingen, I., & Mysterud, A. (2014). Temporal pattern of questing tick Ixodes ricinus density at differing elevations in the coastal region of western Norway. *Parasites & Vectors*, *7*(1), 179. https://doi.org/10.1186/1756-3305-7-179

Räisänen, J., & Alexandersson, H. (2003). A probabilistic view on recent and near future climate change in Sweden. *Tellus A: Dynamic Meteorology and Oceanography*, *55*(2), 113–125.

Randolph, S. E., Green, R. M., Peacey, M. F., & Rogers, D. J. (2000). Seasonal synchrony: The key to tick-borne encephalitis foci identified by satellite data. *Parasitology*, *121*(1), 15–23. https://doi.org/10.1017/S0031182099006083

Randolph, S. E., & Storey, K. (1999). Impact of microclimate on immature tick-rodent host interactions (Acari: Ixodidae): Implications for parasite transmission. *Journal of Medical Entomology*, *36*(6), 741–748.

Rathore, A. P. S., & St. John, A. L. (2020). Cross-Reactive Immunity Among Flaviviruses. *Frontiers in Immunology*, *11*, 334. https://doi.org/10.3389/fimmu.2020.00334

Riccardi, N., Antonello, R. M., Luzzati, R., Zajkowska, J., Di Bella, S., & Giacobbe, D. R. (2019). Tick-borne encephalitis in Europe: A brief update on epidemiology, diagnosis, prevention, and treatment. *European Journal of Internal Medicine*, *62*, 1–6.

Rizzi, J., Nilsen, I. B., Stagge, J. H., Gisnås, K., & Tallaksen, L. M. (2018). Five decades of warming: Impacts on snow cover in Norway. *Hydrology Research*, *49*(3), 670–688.

Roessler, O., Braeuning, A., & Loeffler, J. (2008). Dynamics and driving forces of treeline fluctuation and regeneration in central Norway during the past decades. *Erdkunde*, 117–128.

Ronaghi, M., Shokralla, S., & Gharizadeh, B. (2007). *Pyrosequencing for discovery and analysis of DNA sequence variations*.

Rosenberg, R., Cato, I., Förlin, L., Grip, K., & Rodhe, J. (1996). Marine environment quality assessment of the Skagerrak-Kattegat. *Journal of Sea Research*, *35*(1–3), 1–8.

Ruiz-Fons, F., Fernández-de-Mera, I. G., Acevedo, P., Gortázar, C., & de la Fuente, J. (2012). Factors driving the abundance of Ixodes ricinus ticks and the prevalence of zoonotic I.

ricinus-borne pathogens in natural foci. *Applied and Environmental Microbiology*, *78*(8), 2669–2676.

Ruosteenoja, K., Markkanen, T., & Räisänen, J. (2020). Thermal seasons in northern Europe in projected future climate. *International Journal of Climatology*, *40*(10), 4444–4462.

Ruzek, D., Županc, T. A., Borde, J., Chrdle, A., Eyer, L., Karganova, G., Kholodilov, I., Knap, N., Kozlovskaya, L., & Matveev, A. (2019). Tick-borne encephalitis in Europe and Russia: Review of pathogenesis, clinical features, therapy, and vaccines. *Antiviral Research*, *164*, 23–51.

Sánchez Romano, J., Grund, L., Obiegala, A., Nymo, I. H., Francisco Javier Ancin-Murguzur, Francisco Javier Ancin-Murguzur, Ancin-Murguzur, F. J., Li, H., Hong Li, Li, H., Król, N., Pfeffer, M., & Tryland, M. (2019). A Multi-Pathogen Screening of Captive Reindeer (Rangifer tarandus) in Germany Based on Serological and Molecular Assays. *Frontiers in Veterinary Science*, *6*, 461–461. https://doi.org/10.3389/fvets.2019.00461

Sándor, A. D., Mărcuţan, D. I., D'Amico, G., Gherman, C. M., Dumitrache, M. O., & Mihalca, A. D. (2014). Do the ticks of birds at an important migratory hotspot reflect the seasonal dynamics of Ixodes ricinus at the migration initiation site? A case study in the Danube Delta. *PLoS One*, *9*(2), e89378.

Schley, K., Friedrich, J., Pilz, A., Huang, L., Balkaran, B. L., Maculaitis, M. C., & Malerczyk, C. (2023). Evaluation of under-testing and under-diagnosis of tick-borne encephalitis in Germany. *BMC Infectious Diseases*, *23*(1), 139. https://doi.org/10.1186/s12879-023-08101-6

Schneider, W. L., & Roossinck, M. J. (2001). Genetic Diversity in RNA Virus Quasispecies Is Controlled by Host-Virus Interactions. *Journal of Virology*, *75*(14), 6566–6571. https://doi.org/10.1128/JVI.75.14.6566-6571.2001

Schwaiger, M., & Cassinotti, P. (2003). Development of a quantitative real-time RT-PCR assay with internal control for the laboratory detection of tick borne encephalitis virus (TBEV) RNA. *Journal of Clinical Virology*, *27*(2), 136–145. https://doi.org/10.1016/S1386-6532(02)00168-3

Selås, V. (2006). Explaining bank vole cycles in southern Norway 1980–2004 from bilberry reports 1932–1977 and climate. *Oecologia*, *147*(4), 625–631. https://doi.org/10.1007/s00442-005-0326-7

Selås, V., Framstad, E., Sonerud, G. A., Wegge, P., & Wiig, Ø. (2019). Voles and climate in Norway: Is the abundance of herbivorous species inversely related to summer temperature? *Acta Oecologica*, *95*, 93–99. https://doi.org/10.1016/j.actao.2018.12.002

Shakya, R., Tryland, M., Vikse, R., Romano, J. S., Åsbakk, K., Nymo, I. H., Mehl, R., Evander, M., Ahlm, C., Vapalahti, O., Lwande, O. W., Putkuri, N., Johansen, W., Soleng, A., Edgar, K. S., & Andreassen, Å. K. (2022). Inkoo and Sindbis viruses in blood sucking insects, and a serological study for Inkoo virus in semi-domesticated Eurasian tundra reindeer in Norway. *Virology Journal*, *19*(1), 99. https://doi.org/10.1186/s12985-022-01815-0

Shi, H., Zhou, Y., Jia, E., Pan, M., Bai, Y., & Ge, Q. (2021). Bias in RNA-seq Library Preparation: Current Challenges and Solutions. *BioMed Research International*, *2021*, 1–11. https://doi.org/10.1155/2021/6647597

Sirotkin, M. B., & Korenberg, E. I. (2019). Influence of Abiotic Factors on Infectious Agents Environmentally Associated with Ixodidae Ticks on the Example of Borrelia and Tick-Borne Encephalitis Virus. *Biology Bulletin Reviews*, *9*(6), 543–561. https://doi.org/10.1134/S2079086419060070

Skarpaas, T., Golovljova, I., Vene, S., Ljøstad, U., Sjursen, H., Plyusnin, A., & Lundkvist, Å. (2006). Tickborne Encephalitis Virus, Norway and Denmark. *Emerging Infectious Diseases*, *12*(7), 1136–1138. https://doi.org/10.3201/eid1207.051567

Skarpaas, T., Ljøstad, U., & Sundøy, A. (2004). First Human Cases of Tickborne Encephalitis, Norway. *Emerging Infectious Diseases*, *10*(12), 2241–2243. https://doi.org/10.3201/eid1012.040598

Skarphédinsson, S., Jensen, P. M., & Kristiansen, K. (2005). Survey of Tickborne Infections in Denmark. *Emerging Infectious Diseases*, *11*(7), 1055–1061. https://doi.org/10.3201/eid1107.041265

Skaugen, T. E., & Tveito, O. E. (2004). Growing-season and degree-day scenario in Norway for 2021-2050. *Climate Research*, *26*(3), 221–232.

Slunge, D., Boman, A., & Studahl, M. (2022). Burden of Tick-Borne Encephalitis, Sweden. *Emerging Infectious Diseases*, *28*(2), 314–322. https://doi.org/10.3201/eid2802.204324

Soleng, A., Edgar, K. S., Paulsen, K. M., Pedersen, B. N., B.N. Pedersen, Okbaldet, Y. B., Skjetne, I. E. B., Gurung, D., Vikse, R., & Andreassen, Å. K. (2018). Distribution of Ixodes ricinus ticks and prevalence of tick-borne encephalitis virus among questing ticks in the Arctic Circle region of northern Norway. *Ticks and Tick-Borne Diseases*, *9*(1), 97–103. https://doi.org/10.1016/j.ttbdis.2017.10.002

Sonenshine, D. E. (2005). The biology of tick vectors of human disease. *Tick-Borne Diseases of Humans*, 12–36.

Starodubova, E., Tuchynskaya, K., Kuzmenko, Y., Latanova, A., Tutyaeva, V., Karpov, V., & Karganova, G. (2023). Activation of Early Proinflammatory Responses by TBEV NS1 Varies between the Strains of Various Subtypes. *International Journal of Molecular Sciences*, *24*(2), 1011. https://doi.org/10.3390/ijms24021011

Statens Serum Institut. (2020). *TBE report on disease occurrence 2017-2019*. Statens Serum Institut. https://en.ssi.dk/surveillance-and-preparedness/surveillance-in-denmark/annual-reports-on-disease-incidence/tbe-report-on-disease-occurrence-2017-2019

Statens Serum Institut. (2023). *Annual report on TBE (Central European encephalitis) in Denmark, 2020-2022* [Government]. https://en.ssi.dk/news/epi-news/2023/no-23---2023

Stegmüller, S., Fraefel, C., & Kubacki, J. (2023). Genome Sequence of Alongshan Virus from Ixodes ricinus Ticks Collected in Switzerland. *Microbiology Resource Announcements*, *12*(3), e01287-22. https://doi.org/10.1128/mra.01287-22

Stenseth, N. C., VIljugrein, H., Jędrzejewski, W., Mysterud, A., & Pucek, Z. (2002). Population dynamics ofClethrionomys glareolus andApodemus flavicollis: Seasonal components of density dependence and density independence. *Acta Theriologica*, *47*(1), 39–67. https://doi.org/10.1007/BF03192479

Stiasny, K., Malafa, S., Aberle, S. W., Medits, I., Tsouchnikas, G., Aberle, J. H., Holzmann, H., & Heinz, F. X. (2021). Different Cross-Reactivities of IgM Responses in Dengue, Zika and Tick-Borne Encephalitis Virus Infections. *Viruses*, *13*(4), 596. https://doi.org/10.3390/v13040596

Stjernberg, L., Holmkvist, K., & Berglund, J. (2008). A newly detected tick-borne encephalitis (TBE) focus in south-east Sweden: A follow-up study of TBE virus (TBEV) seroprevalence. *Scandinavian Journal of Infectious Diseases*, *40*(1), 4–10. https://doi.org/10.1080/00365540701522934

Su, J., Andrée, E., Nielsen, J. W., Olsen, S. M., & Madsen, K. S. (2021). Sea Level Projections From IPCC Special Report on the Ocean and Cryosphere Call for a New Climate

Adaptation Strategy in the Skagerrak-Kattegat Seas. *Frontiers in Marine Science*, *8*, 629470. https://doi.org/10.3389/fmars.2021.629470

Suppan, J., Engel, B., Marchetti-Deschmann, M., & Nürnberger, S. (2018). Tick attachment cement–reviewing the mysteries of a biological skin plug system. *Biological Reviews*, *93*(2), 1056–1076.

Süss, J. (2003). Epidemiology and ecology of TBE relevant to the production of effective vaccines. *Vaccine*, *21*, S19–S35. https://doi.org/10.1016/S0264-410X(02)00812-5

Süss, J. (2011). Tick-borne encephalitis 2010: Epidemiology, risk areas, and virus strains in Europe and Asia—An overview. *Ticks and Tick-Borne Diseases*, *2*(1), 2–15.

Svensson, J., Christiansen, C. B., & Persson, K. E. M. (2021). A Serosurvey of Tick-Borne Encephalitis Virus in Sweden: Different Populations and Geographical Locations. *Vector-Borne and Zoonotic Diseases*, *21*(8), 614–619. https://doi.org/10.1089/vbz.2020.2763

Tack, W., Madder, M., Baeten, L., Vanhellemont, M., Gruwez, R., & Verheyen, K. (2012). Local habitat and landscape affect Ixodes ricinus tick abundances in forests on poor, sandy soils. *Forest Ecology and Management*, *265*, 30–36.

Tagliapietra, V., Rosà, R., Arnoldi, D., Cagnacci, F., Capelli, G., Montarsi, F., Hauffe, H., & Rizzoli, A. (2011). Saturation deficit and deer density affect questing activity and local abundance of Ixodes ricinus (Acari, Ixodidae) in Italy. *Veterinary Parasitology*, *183*(1–2), 114–124.

Tälleklint, L., & Jaenson, T. G. (1998). Increasing geographical distribution and density of Ixodes ricinus (Acari: Ixodidae) in central and northern Sweden. *Journal of Medical Entomology*, *35*(4), 521–526.

The Danish Government. (2008). *Danish strategy for adaptation to a changing climate* ([1. oplag]). Danish Energy Agency.

Thoman, R. L., Druckenmiller, M. L., Moon, T. A., Andreassen, L., Baker, E., Ballinger, T. J., Berner, L. T., Bernhard, G. H., Bhatt, U. S., & Bjerke, J. W. (2022). The Arctic. *Bulletin of the American Meteorological Society*, *103*(8), S257–S306.

Tokarevich, N. K., Tronin, A. A., Blinova, O. V., Buzinov, R. V., Boltenkov, V. P., Yurasova, E. D., & Nurse, J. (2011a). The impact of climate change on the expansion of *Ixodes persulcatus* habitat and the incidence of tick-borne encephalitis in the north of European Russia. *Global Health Action*, *4*(1), 8448. https://doi.org/10.3402/gha.v4i0.8448

Tokarevich, N. K., Tronin, A. A., Blinova, O. V., Buzinov, R. V., Boltenkov, V. P., Yurasova, E. D., & Nurse, J. (2011b). The impact of climate change on the expansion of *Ixodes persulcatus* habitat and the incidence of tick-borne encephalitis in the north of European Russia. *Global Health Action*, *4*(1), 8448. https://doi.org/10.3402/gha.v4i0.8448

Tokarevich, N., Tronin, A., Gnativ, B., Revich, B., Blinova, O., & Evengard, B. (2017). Impact of air temperature variation on the ixodid ticks habitat and tick-borne encephalitis incidence in the Russian Arctic: The case of the Komi Republic. *International Journal of Circumpolar Health*, *76*(1), 1298882. https://doi.org/10.1080/22423982.2017.1298882

Tonteri, E., Jokelainen, P., Matala, J., Pusenius, J., & Vapalahti, O. (2016). Serological evidence of tick-borne encephalitis virus infection in moose and deer in Finland: Sentinels for virus circulation. *Parasites & Vectors*, *9*(1), 54–54. https://doi.org/10.1186/s13071-016-1335-6

Traavik, T. (1979). Antibodies to Tick-Borne Encephalitis Virus in Human Sera from the Western Coast of Norway. *Acta Pathologica Microbiologica Scandinavica Section B Microbiology, 87*(1-6), 9–13.

Traavik, T., Mehl, R., & Wiger, R. (1978). The first tick-borne encephalitis virus isolates from Norway. *Acta Pathologica Microbiologica Scandinavica Section B Microbiology*, *86*(1-6), 253–256.

Truong, C., Palmé, A. E., & Felber, F. (2007). Recent invasion of the mountain birch Betula pubescens ssp. Tortuosa above the treeline due to climate change: Genetic and ecological study in northern Sweden. *Journal of Evolutionary Biology, 20*(1), 369–380.

Van Oort, B. E. H., Hovelsrud, G. K., Risvoll, C., Mohr, C. W., & Jore, S. (2020). A Mini-Review of Ixodes Ticks Climate Sensitive Infection Dispersion Risk in the Nordic Region. *International Journal of Environmental Research and Public Health*, *17*(15), 5387. https://doi.org/10.3390/ijerph17155387

Vanneste, T., Michelsen, O., Graae, B. J., Kyrkjeeide, M. O., Holien, H., Hassel, K., Lindmo, S., Kapás, R. E., & De Frenne, P. (2017). Impact of climate change on alpine vegetation of mountain summits in Norway. *Ecological Research*, *32*(4), 579–593. https://doi.org/10.1007/s11284-017-1472-1

Vikse, R., Paulsen, K. M., Edgar, K. S., Pettersson, J. H.-O., Ottesen, P., Okbaldet, Y. B., Kiran, N., Lamsal, A., Lindstedt, H. E. H., Pedersen, B. N., Soleng, A., & Andreassen, Å. K. (2020). Geographical distribution and prevalence of tick-borne encephalitis virus in questing Ixodes ricinus ticks and phylogeographic structure of the Ixodes ricinus vector in Norway. *Zoonoses and Public Health*, *67*(4), 370–381. https://doi.org/10.1111/zph.12696

Waldeck, M., Winqvist, N., Henriksson, G., Dyrdak, R., Settergren, B., & Lindgren, P.-E. (2022). Surveillance of tick-borne encephalitis in emerging risk areas in southern Sweden: A retrospective case finding study. *European Journal of Clinical Microbiology & Infectious Diseases*. https://doi.org/10.1007/s10096-022-04509-1

Waldeck, M., Winqvist, N., Henriksson, G., Dyrdak, R., Settergren, B., & Lindgren, P.-E. (2023). Surveillance of tick-borne encephalitis in emerging risk areas in southern Sweden: A retrospective case finding study. *European Journal of Clinical Microbiology & Infectious Diseases*, *42*(1), 13–22. https://doi.org/10.1007/s10096-022-04509-1

Waldenström, J., Lundkvist, Å., Falk, K. I., Garpmo, U., Bergström, S., Lindegren, G., Lindegren, G., Sjöstedt, A., Mejlon, H., Fransson, T., Haemig, P. D., & Olsen, B. R. (2007). Migrating birds and tickborne encephalitis virus. *Emerging Infectious Diseases*, *13*(8), 1215–1218. https://doi.org/10.3201/eid1308.061416

Wallenhammar, A., Lindqvist, R., Asghar, N., Gunaltay, S., Fredlund, H., Davidsson, Å., Andersson, S., Överby, A. K., & Johansson, M. (2020). Revealing new tick-borne encephalitis virus foci by screening antibodies in sheep milk. *Parasites & Vectors*, *13*(1), 185. https://doi.org/10.1186/s13071-020-04030-4

Wilhelmsson, P., Jaenson, T. G. T., Olsen, B., Waldenström, J., & Lindgren, P.-E. (2020). Migratory birds as disseminators of ticks and the tick-borne pathogens Borrelia bacteria and tick-borne encephalitis (TBE) virus: A seasonal study at Ottenby Bird Observatory in South-eastern Sweden. *Parasites & Vectors*, *13*(1), 607. https://doi.org/10.1186/s13071-020-04493-5

Yun, S.-M., Kim, S. Y., Ju, Y. R., Han, M. G., Jeong, Y. E., & Ryou, J. (2011). First complete genomic characterization of two tick-borne encephalitis virus isolates obtained from wild rodents in South Korea. *Virus Genes*, *42*(3), 307–316. https://doi.org/10.1007/s11262-011-0575-y

Zając, Z., Kulisz, J., Bartosik, K., Woźniak, A., Dzierżak, M., & Khan, A. (2021). Environmental determinants of the occurrence and activity of Ixodes ricinus ticks and the prevalence of tick-borne diseases in eastern Poland. *Scientific Reports*, *11*(1), 15472. https://doi.org/10.1038/s41598-021-95079-3

Zakotnik, S., Knap, N., Bogovič, P., Zorec, T. M., Poljak, M., Strle, F., Avšič-Županc, T., & Korva, M. (2022). Complete Genome Sequencing of Tick-Borne Encephalitis Virus Directly from Clinical Samples: Comparison of Shotgun Metagenomic and Targeted Amplicon-Based Sequencing. *Viruses*, *14*(6), 1267. https://doi.org/10.3390/v14061267

Zeman, P., & Beneš, C. (2004). A tick-borne encephalitis ceiling in Central Europe has moved upwards during the last 30 years: Possible impact of global warming? *International Journal of Medical Microbiology Supplements*, *293*, 48–54.

Paper I

Lamsal, A., Edgar, K. S., Jenkins, A., Renssen, H., Kjær, L. J., Alfsnes, K., Bastakoti, S., Dieseth, M., Klitgaard, K., Lindstedt, H. E. H., Paulsen, K. M., Vikse, R., Korslund, L., Kjelland, V., Stuen, S., Kjellander, P., Christensson, M., Teräväinen, M., Jensen, L. M., Bødker, R., Soleng, A., Andreassen, Å. K. (2023). Prevalence of tick-borne encephalitis virus in questing *Ixodes ricinus* nymphs in southern Scandinavia and the possible influence of meteorological factors. *Zoonoses and Public Health*, 70, 473–484. https://doi.org/10.1111/zph.13049

Received: 1 March 2023 | Revised: 25 April 2023 | Accepted: 6 May 2023

DOI: 10.1111/zph.13049

ORIGINAL ARTICLE

WILEY

Prevalence of tick-borne encephalitis virus in questing *Ixodes ricinus* nymphs in southern Scandinavia and the possible influence of meteorological factors

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Funding information
ScandTick, Grant/Award Number: 167226; ScandTick Innovation, Grant/Award Number: 20200422

Abstract

Ixodes ricinus ticks are Scandinavia's main vector for tick-borne encephalitis virus (TBEV), which infects many people annually. The aims of the present study were (i) to obtain information on the TBEV prevalence in host-seeking *I. ricinus* collected within the Øresund-Kattegat-Skagerrak (ØKS) region, which lies in southern Norway, southern Sweden and Denmark; (ii) to analyse whether there are potential spatial patterns in the TBEV prevalence; and (iii) to understand the relationship between TBEV

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prevalence and meteorological factors in southern Scandinavia. Tick nymphs were collected in 2016, in southern Scandinavia, and screened for TBEV, using pools of 10 nymphs, with RT real-time PCR, and positive samples were confirmed with pyrosequencing. Spatial autocorrelation and cluster analysis was performed with Global Moran's *I* and SatScan to test for spatial patterns and potential local clusters of the TBEV pool prevalence at each of the 50 sites. A climatic analysis was made to correlate parameters such as minimum, mean and maximum temperature, relative humidity and saturation deficit with TBEV pool prevalence. The climatic data were acquired from the nearest meteorological stations for 2015 and 2016. This study confirms the presence of TBEV in 12 out of 30 locations in Denmark, where six were from Jutland, three from Zealand and two from Bornholm and Falster counties. In total, five out of nine sites were positive from southern Sweden. TBEV prevalence of 0.7%, 0.5% and 0.5%, in nymphs, was found at three sites along the Oslofjord (two sites) and northern Skåne region (one site), indicating a potential concern for public health. We report an overall estimated TBEV prevalence of 0.1% in questing *I. ricinus* nymphs in southern Scandinavia with a region-specific prevalence of 0.1% in Denmark, 0.2% in southern Sweden and 0.1% in southeastern Norway. No evidence of a spatial pattern or local clusters was found in the study region. We found a strong correlation between TBEV prevalence in ticks and relative humidity in Sweden and Norway, which might suggest that humidity has a role in maintaining TBEV prevalence in ticks. TBEV is an emerging tick-borne pathogen in southern Scandinavia, and we recommend further studies to understand the TBEV transmission potential with changing climate in Scandinavia.

KEYWORDS
climate change, flaviviruses, *I. ricinus*, Nordic, tick-borne encephalitis virus

1 | INTRODUCTION

The incidence of tick-borne encephalitis (TBE) has been increasing in Sweden, Norway and Denmark in the last few decades (Jaenson et al., 2018; MSIS, 2022; Public Health Agency of Sweden, 2021; Slunge et al., 2022; Statens Serum Institut, 2020). Sweden had the highest incidence of human cases, with 520 cases in 2021 (Public Health Agency of Sweden, 2021). The number of reported TBE cases in Norway has doubled from 2020 to 2021, with 72 cases reported in 2021 (MSIS, 2022). The incidence of human TBE cases in Denmark has also increased, where 13 TBE cases, the highest number until now, were reported in 2019 (Statens Serum Institut, 2020). TBE is caused by different geographically distributed subtypes of tick-borne encephalitis virus (TBEV). Five subtypes of TBEV have been identified so far: the European (TBEV-Eu), Siberian (TBEV-Sib), Far Eastern (TBEV-Fe), Himalayan (TBEV-Him) and Baikalian (TBEV-Bkl) (Dai et al., 2018; Kovalev & Mukhacheva, 2017). In Scandinavia, *I. ricinus* is the most common tick species and is the primary vector for the European subtype of TBEV (TBEV-Eu). The virus is transmitted to humans by tick bites, mainly by nymphs, and occasionally through the consumption of unpasteurized dairy products (Caini et al., 2012; Hudopisk et al., 2013). TBEV has also been detected in raw milk from cows and sheep (Cisak et al., 2010; Paulsen et al., 2019; Wallenhammar et al., 2020). TBEV infection may vary from being asymptomatic to fatal meningitis, encephalitis,

Impacts

- This is one of the most extensive studies aimed to describe the prevalence of TBEV in southern Scandinavia, screening 29,570 questing *Ixodes ricinus* nymphs.
- The virus was detected at 20 out of 50 locations, demonstrating that it is widespread in this region. The study confirms the presence of TBEV in ticks in Denmark, with a higher prevalence in Norway's Oslofjord and Sweden's southern Skåne County.
- This study also highlights the possible influence of relative humidity in sustaining TBEV in the region. TBEV was detected from regions with and without previously reported human TBE cases, which is highly relevant information for public health considerations and risk evaluation.

meningoencephalitis and meningoencephalomyelitis (Kaiser, 1999); however, infections with TBEV-Eu are often reported to be asymptomatic (Gritsun et al., 2003; Larsen et al., 2014; Marvik et al., 2021; Svensson et al., 2021; Thortveit et al., 2020).TBE has been a notifiable disease in Sweden and Norway since 1969 and 1994, respectively, but is not at present notifiable in Denmark.

In Southern Scandinavia, the spatial distribution of TBE cases does not fully coincide with the known distribution of TBEV in ticks. TBEV has been detected in ticks from coastal counties in Norway, from Viken in the southeast to Nordland in the north (Andreassen et al., 2012; Paulsen et al., 2015; Soleng et al., 2018; Vikse et al., 2020). However, all human TBE cases from Norway are reported from the southernmost parts of the country, specifically from the counties Agder, Vestfold and Telemark, and Viken (former Buskerud) (MSIS, 2022). The south-western coast of the Oslofjord is a known endemic area with several reported cases, while there are few reported cases on the eastern side, although TBEV has been detected in ticks within the eastern side since 2015 (Larsen et al., 2014; Marvik et al., 2021; MSIS, 2022; Vikse et al., 2020). Studies report TBEV seroprevalence in healthy blood donors from both the eastern and western sides of the Oslofjord with seroprevalence of 0.65% and 0.4%, respectively, suggesting that TBEV infections within the population might have been asymptomatic or undiagnosed (Larsen et al., 2014; Marvik et al., 2021). TBE might not have been diagnosed due to a lack of awareness of tick bites and the distribution range of the virus (Paulsen et al., 2015).

Tick-borne encephalitis virus may be present at very low, potentially undetectable concentrations in questing ticks but might replicate and become detectable under favourable conditions in engorged ticks (Belova et al., 2012; Pettersson et al., 2014). This might explain the lack of correspondence between TBEV sentinel studies and TBEV prevalence estimation from ticks. In Denmark, although TBEV seropositive deer are reported from multiple sites (Andersen, Bestehorn, et al., 2019; Andersen, Larsen, et al., 2019; Skarphédinsson et al., 2005), several tick pools collected in 2010–2011 from northern Zealand, Funen and Jutland were negative for TBEV (Fomsgaard, 2020). Very few human cases have been reported in Denmark, mostly from the Island of Bornholm and sporadic cases from northern Zealand (Agergaard et al., 2019; Fomsgaard et al., 2009). Locally acquired human TBE cases were reported from Jutland and southern Funen in 2018 and one from the island of Falster in 2020.

Sweden has wider known TBEV endemic areas from where human TBE cases, seropositive cervids and TBEV prevalence in ticks have been reported (Jaenson et al., 2012, 2018; Jaenson & Wilhelmsson, 2019; Lundkvist et al., 2011; Pettersson et al., 2014; Wilhelmsson et al., 2020). Human TBE cases are widely reported from the coastal and central areas of Sweden around Stockholm, Örebro and the western Götaland region. The human TBE cases are typically reported from places with high population densities, such as the Swedish capital of Stockholm and lately from around the great Swedish lakes, where TBEV in ticks previously has been reported (Brinkley et al., 2008; Melik et al., 2007).In the later 2010s, studies reported TBEV endemic foci from inland central Sweden to southern and western Sweden (Brinkley et al., 2008; Stjernberg et al., 2008). The virus has been detected in ticks up to Norrbotten in the Gulf of Bothnia in northern Sweden (Jaenson & Wilhelmsson, 2019; Pettersson et al., 2014). However, TBEV prevalence estimation in ticks is still limited in southernmost Sweden (Pettersson et al., 2014).

Tick-borne encephalitis virus primarily circulates among tick populations and rodent hosts that act as reservoirs to persist in the environment (Michelitsch et al., 2019). Climatic factors, such as temperature, humidity, snow cover and rainfall, can influence the distribution of ticks and their hosts, potentially leading to the expansion of TBEV's geographical range as a result of climate change (Jaenson et al., 2012). TBEV transmission in ticks is attributed to tick seasonal activity, which is dependent on the tick life cycle (Randolph et al., 2000). Ticks can acquire infection by feeding on viremic hosts, mostly rodents (Achazi et al., 2011). Larvae can be infected with TBEV from infected adult females through eggs known as transovarial transmission. Mathematical models have shown that when larvae and nymphs feed in close proximity, nonviremic transmission of TBEV can occur from infected to uninfected ticks known as cofeeding transmission (Randolph, 2011; Randolph et al., 1996). This has been identified as a major route of TBEV transmission within a tick population in eastern Europe (Nah & Wu, 2021). Once infected with TBEV, a tick remains infected throughout its life (Jaenson et al., 2012; Kozuch & Nosek, 1980). The prevalence of TBEV in ticks depends on a variety of other factors such as the availability of hosts for feeding opportunities, the type of vegetation for suitable moulting environments and opportunities for transboundary transmission via for example migratory birds. Studies have reported a higher tick abundance in forested areas compared with open meadow areas, due to humid conditions found in forested areas, protecting against desiccation, while open habitats are more exposed to the effects of sun and wind (Jaenson et al., 2018; Lindström & Jaenson, 2003; Medlock et al., 2013). The increase in tick host species, for example, roe deer (*Capreolus capreolus*), has been suggested to cause the increase in human TBE incidence in Sweden (Jaenson et al., 2012). Other studies have found TBEV-infected ticks on migratory birds (Kazarina et al., 2015; Waldenström et al., 2007) thus, migrating birds may play a role in the geographical dispersal of TBEV-infected ticks, and their potential to start a new TBEV foci (Waldenström et al., 2007).

The vector, *I. ricinus* ticks are greatly influenced by temperature and show analogous seasonal variation; however, very less is known about the variation of TBEV and its relationship with the prevailing microclimate (Daniel et al., 2018). The vegetation period has been prolonged in Scandinavia, which has been identified as a key factor contributing to the increased abundance and activity of ticks in Sweden and Norway (Hvidsten et al., 2020; Jaenson et al., 2012). The impacts of climate change in Nordic countries are estimated by wetter and warmer climate with an increase in the length of growing season in the future (Hanssen-Bauer et al., 2017; Randolph, 2001). Although *I. ricinus* may expand its northern range in Scandinavia with climatic changes, it was also found that a potential range expansion in Scandinavia would only affect a small additional fraction of the human population as most of the population already live close to established tick areas (Kjær et al., 2019). TBEV poses a health risk to people living close to areas with TBEV presence in ticks (Vikse et al., 2020). TBE is a climate-sensitive disease, and the risk of virus transmission can be influenced by environmental factors such as temperature, humidity and saturation deficit (Jaenson et al., 2012; Medlock et al., 2013; Randolph et al., 2000). It is important to note that the direct effect of temperature and humidity conditions on TBEV is practically unknown (Korenberg, 2009). It is usually agreed that microorganisms in ticks are generally well adapted to the variability of temperature and humidity as well as other environmental conditions that are important for reproduction, vertical and horizontal transmission (Sirotkin & Korenberg, 2019). Although the TBEV infection in ticks is affected by both temperature and relative humidity (RH), RH has been suggested to be the major determinant for infection rate rather than temperature (Danielová et al., 1983). This highlights the importance of identifying regions with high pathogen prevalence. This study aims to provide updated knowledge on TBEV distribution and prevalence in ticks in relation to climatic parameters in southern Norway, southern Sweden and Denmark.

2 | MATERIALS AND METHODS

2.1 | Tick collection and stratification of study sites

We collected ticks at each site between 15 August and 30 September 2016, from 11 sites along the Oslofjord and southern Norway, 30 sites in Denmark and nine sites in southern Sweden as part of the ScandTick Innovation project. Ticks were collected from areas below 450 m above sea level (masl). A total of 29,570 tick nymphs were collected according to the tick identification key by Hillyard (1996) while larvae and adult ticks were excluded from the analysis. The collected nymphs were pooled in groups of 10, totaling 2957 tick pools. The procedures for vegetation stratification, site selection, tick collection and storage have been described in previous studies (Jung Kjær et al., 2019; Kjær et al., 2019, 2020). In short, each country within the study region was divided into a northern and southern part (of equal sizes) and furthermore divided into high and low values of the maximum normalized difference vegetation index (NDVI, from Fourier processed satellite imagery 40). Lastly, these stratified regions were further divided into forest and meadow, using Corine land cover (all one × 1 km resolution 40), based on the Corine definitions; forest: broad-leaved forest, coniferous forest, mixed forest and meadow: land principally occupied by agriculture with significant areas of natural vegetation, natural grasslands, moors and heathland, transitional woodland-shrub (Jung Kjær et al., 2019; Kjær et al., 2019, 2020). Tick collection sites were then selected at random within the stratified regions (80% forest and 20% meadow).

2.2 | Laboratory methods

2.2.1 | TBEV detection

Tick-borne encephalitis virus was detected as previously described by Andreassen et al. (2012). Briefly, ticks were homogenized as described previously by Klitgaard et al. (2019) and sent to the Norwegian Institute of Public Health on dry ice. The samples were stored at −80°C until further analysis. Total RNA was extracted from homogenized nymphs using the RNeasy mini kit (QIAGEN Inc.) with an automated QIAcube instrument (Qiagen). Immediately after the extraction process, the RNA was reversely transcribed to cDNA with random primers (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems). An in-house real-time reverse transcriptase RT-PCR was performed targeting a 54-base pair (bp) fragment on the envelope gene of TBEV. As positive controls, RNA from the TBEV strains 'Soukup' or 'Hochosterwitz' (kindly provided by Christian Beuret, Spiez lab, Switzerland and Franz-Xaver Heinz, University of Vienna, Austria, respectively) were used, and nuclease-free water was used as a negative control. All the RT-PCR-positive TBEV pools were pyro-sequenced and compared with a positive control for confirmation as described earlier (Andreassen et al., 2012). A pyro run was deemed valid when all TBEV controls were positive, all water controls were negative, and PCR-positive pools show pyrogram plots, which followed the positive control pattern. PCR-positive pools that could not be confirmed by pyrosequencing were excluded from the prevalence calculation. A few PCR-positive samples could not be confirmed by pyrosequencing due to technical errors. These comprised 8 tick pools from Denmark. The total number of pools included in the study was 2957 (1790 from Denmark, 660 from Norway and 507 from Sweden).

2.2.2 | TBEV prevalence in ticks

We calculated Estimated Pooled Prevalence (EPP) using the online Epitools epidemiological calculator with fixed pooled size and perfect test (https://epitools.ausvet.com.au/ppfreqone). We used EPP in all further analysis except for the spatial analyses. To test for differences in TBEV pool prevalence between sites, we used Pearson's chi-squared test statistics (test of equal or given proportions). This test is nonspatial and only tests whether there is a statistically significant difference between the site-specific pool prevalences.

2.3 | Spatial analyses

We tested Global clustering, in the context of a regional study 'global clustering refers to the identification of larger-scale patterns or trends within the study area', by Global Moran's *I* to test spatial patterns between the TBEV pool prevalence at each of the

TABLE 1 Tick-borne encephalitis virus (TBEV) in *Ixodes ricinus* tick nymphs in southern Scandinavia.

Country	Number of PCR-positive pools/ total tick pools analysed	Positive sites/ total sites tested	Number of confirmed TBEV-positive pools by pyrosequencing	Estimated pooled prevalence (%) by country
Southern Norway	10/660	3/11	8	0.1 (0.1–0.2)
Southern Sweden	11/507	5/9	8	0.2 (0.1–0.3)
Denmark	21/1790	12/30	13	0.1 (0.1–1.5)
Total	42/2957	20/50	29	0.1 (0.1–0.2)

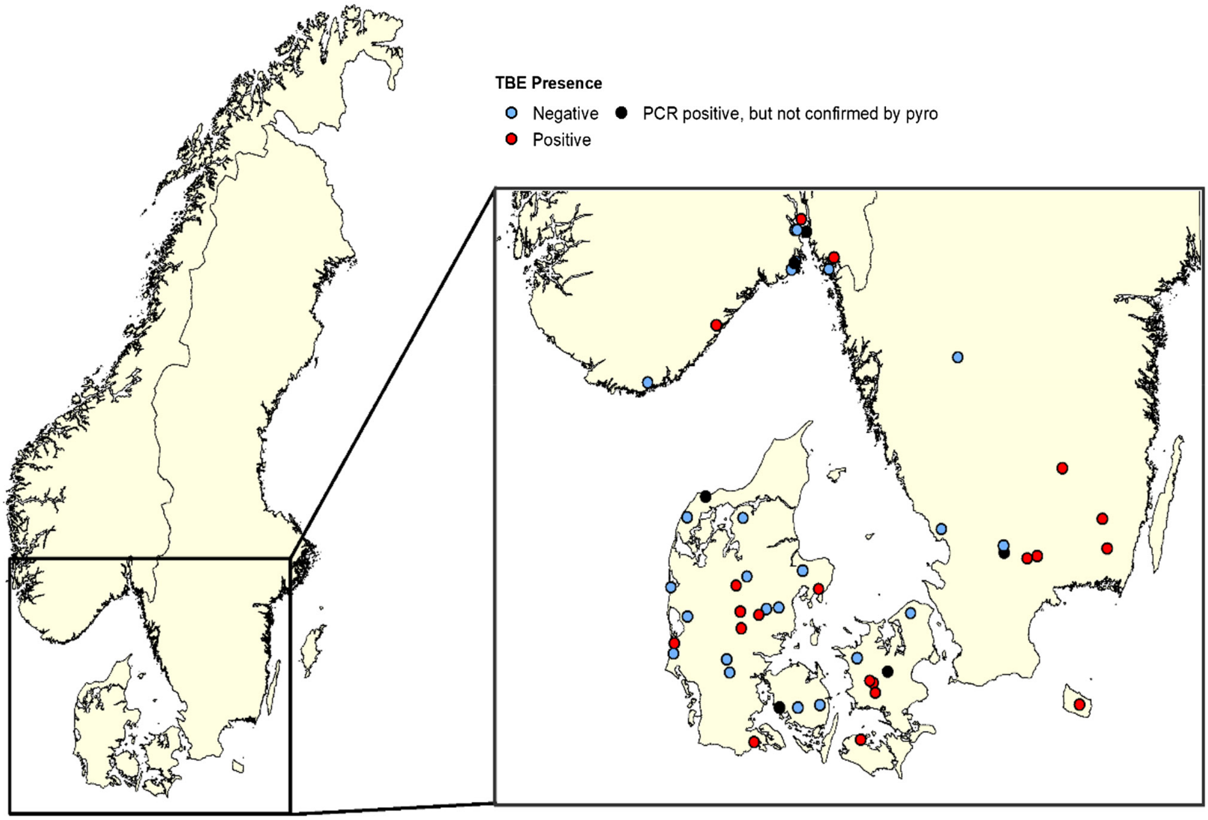

FIGURE 1 Visualization of TBEV-positive areas in Denmark, southern Norway and southern Sweden. Areas with unconfirmed TBEV-positive sample are marked in black, blue dots are negative sites and red dots are positive sites.

50 sites. Global Moran's *I* measure spatial autocorrelation based on site location and the TBEV prevalence at the sites and evaluates whether the observed prevalence patterns are clustered, dispersed or random.

To identify potential local clusters of TBEV within the study region, we used the program SatScan (Kulldorff, 2018) and the package *rsatscan* (Kleinman, 2015) in R 3.5.2 (R Development Core Team, 2018). We selected an elliptical scanning window and the Bernoulli probability model along with a maximum spatial window size of less than or equal to 50% of the total population at risk. The analysis looks for significant geographical clusters within circular or ellipsoid areas and tests whether EPP at sites included in the cluster on average has higher (hotspots) or lower (cold spots) prevalence compared with sites outside the clusters. The relative risk was used as a measure of clustering, which is the estimated risk within the cluster divided by the estimated risk outside the cluster. We used the Gini coefficient (Han et al., 2016) to evaluate the clusters, which measures the heterogeneity of the clusters, and thus determines whether to report multiple smaller clusters or a large joint cluster. In this analysis, we used the observed proportion of TBEV (RT real-time-PCR) positive pools rather than the estimated pooled prevalence (EPP). Before running the analysis, we transformed site coordinates into a flat UTM projection (UTM zone 32 N).

2.4 | Climatic analysis

We obtained climatic data from the Norwegian Meteorological Institute (www.met.no/), the Swedish Meteorological and Hydrological Institute (www.smhi.se) and the Danish Meteorological Institute (www.dmi.dk/) and compiled the data over the period 2015–2016. We chose data from weather stations, based on the closest distance to the sampling site. From these weather stations, we acquired air temperatures (°C) and RH (%) along with their monthly extremes (mean, maximum and minimum) and we calculated saturation deficit (SD), which is the measurement of drying power of the air according to Randolph and Storey (1999) (Andreassen et al., 2012; Estrada-Peña et al., 2004; Perret et al., 2004; Randolph & Storey, 1999). Months were grouped into the following seasons: March, April and May were considered Spring; June and July were considered Summer; August, September and October were considered Autumn and November, December, January and February were considered Winter. A Pearson's Correlation analysis was conducted to examine the correlation between the EPP and several meteorological factors: temperature, RH and SD, as well as the extremes of these factors. This analysis included only TBEV-positive sites and classified the variables according to country, season and year. The analysis was conducted using SPSS version 22, and statistical significance was assessed at either $p < 0.05$ or $p < 0.01$.

3 | RESULTS

3.1 | TBEV prevalence in southern Scandinavia

Of the 50 sites tested, 20 (40% of sites) were found to be positive for TBEV (Table 1). In total, we tested 2957 tick pools of which 29 pools were positive resulting in an overall TBEV prevalence of 0.1% in questing *I. ricinus* nymphs in southern Scandinavia (Figure 1, Table 2). The nonspatial Pearson's chi-squared test statistics testing for differences in EPP between sites was statistically significant ($\chi^2_{49} = 70.9$, $p = 0.02$) and thus not randomly distributed. All the TBEV-positive pools came from forested habitats.

3.2 | TBEV prevalence in tick nymphs from southern Sweden

In southern Sweden, five of nine sites were positive for TBEV. A large proportion of the positive pools (five out of eight) came from two sites in northern Skåne County located only 11 km apart. Blekinge, Kalmar and Jönköping Counties all had one positive site. Among the two real-time RT-PCR-positive pools from Blekinge, one was lost due to technical errors during pyrosequencing. The overall TBEV prevalence in ticks in southern Sweden was 0.2%.

3.3 | TBEV prevalence in tick nymphs from southern Norway

Tick-borne encephalitis virus was detected in eight pools out of 660 pools tested giving an EPP of 0.1% in southern Norway. A total of three of 11 sites were positive for TBEV and two of those sites from Oslofjord showed a relatively high EPP of 0.7% and 0.5%, respectively (Table 2). One positive tick pool came from the coastal area of Agder County in the southern part of Norway.

3.4 | TBEV prevalence in tick nymphs from Denmark

In Denmark, a total of 12 of 30 sites were positive and 13 tick pools out of 1790 were positive, resulting in an overall TBEV prevalence of 0.1% (Table 1). Seven of the positive sites were from Jutland, three from Zealand, one from Falster and one from Bornholm. Among the 12 positive sites, 11 sites had one positive pool, while the last location from southern Denmark, close to the North Sea, had two positive pools (Figure 1, Table 2). We lost five positive pools from three additional sites in Denmark during pyrosequencing (shown by black-filled dots, Figure 1). These contained two pools from one site on Funen, two pools from one site from western Zealand and one pool from one site from northern Jutland (Table 2). We also lost three pools out of four from one site on Bornholm, which site is included in the 12 positive sites above.

3.5 | Spatial patterns in TBEV prevalence

We found no evidence of global clustering (Moran's $I = 0.017$, $z = 0.38$, $p = 0.71$) for site-specific pool prevalence, and none of the local clusters found within the study region using SatScan were statistically significant ($p > 0.1$). The results show that the amount of prevalence varied between the sites, but it did not seem to be grouped in any specific area within the region.

3.6 | Correlation analysis of TBEV prevalence in ticks with meteorological factors

We used Pearson's correlation analysis to investigate the relationship between EPP with meteorological factors on data from the autumn and winter of 2015 and spring, summer and autumn of 2016. The meteorological factors included mean, minimum and maximum of temperature; mean, minimum and maximum of RH; and mean, minimum and maximum of SD during the autumn and winter of 2015 and spring, summer and autumn of 2016.

We found a statistically significant correlation between the EPP and RH in Sweden and Norway. There was a positive relationship between EPP and monthly minimum RH in all seasons in Sweden: autumn of 2016 (df = 13, $r = 0.7$, $p = 0.01$), winter (df = 18, $r = 0.6$, $p = 0.00$), spring (df = 13, $r = 0.6$, $p = 0.02$), summer (df = 8, $r = 0.63$, $p = 0.05$) and autumn (df = 13, $r = 0.58$, $p = 0.02$). This relationship was also present for mean RH in winter 2015 (df = 18, $r = 0.7$, $p = 0.01$) and autumn 2016 (df = 13, $r = 0.6$, $p = 0.02$) (Table S1). In Norway, the EPP showed statistically significant negative correlation with maximum RH and statistically significant positive correlation with minimum SD in the spring, summer and autumn of 2016. Specifically, in spring (df = 7, $r = -0.9$, $p = 0.00$; df = 7, $r = 0.8$, $p = 0.00$), summer (df = 4, $r = -0.9$, $p = 0.00$; df = 4, $r = 0.7$, $p = 0.01$) and autumn (df = 7, $r = -0.9$, $p = 0.00$; df = 7, $r = 0.9$, $p = 0.00$), there was a strong negative relationship with monthly maximum RH and a strong positive

TABLE 2 Detailed description of the sites where TBEV was detected in host-seeking *Ixodes ricinus* nymphs in southern Scandinavia.

Country	County	SiteID	Longitude	Latitude	Habitat type	No. of PCR-positive pools/pools tested	No. of confirmed PCR pools[a]	EPP% (range of EPP)
Norway	Vestfold and Telemark	NO-124	8.92	58.61	Forest, high NDVI	1/60	1	0.2 (0.0–0.9)
Norway	Viken	O-111	11.1	59.19	Forest, high NDVI	4/60	3	0.5 (0.10–1.5)
Norway	Viken	O-238	10.51	59.53	Forest, low NDVI	4/60	4	0.7 (0.20–1.8)
Denmark	Jutland	DK-002	8.22	55.79	Forest, low NDVI	2/60	2	0.3 (0.0–1.2)
Denmark	Zealand	DK-025	11.55	55.42	Forest, low NDVI	1/60	1	0.2 (0.0–0.9)
Denmark	Falster	DK-120	9.56	54.92	Forest, high NDVI	1/60	1	0.2 (0.0–0.9)
Denmark	Zealand	DK-121	11.59	55.33	Forest, high NDVI	1/60	1	0.2 (0.0–0.9)
Denmark	Bornholm	DK-123	14.96	55.11	Forest, high NDVI	4/60	1, 3 lost[b]	0.2 (0.0–0.9)
Denmark	Zealand	DK-124	11.5	55.44	Forest, high NDVI	1/60	1	0.2 (0.0–0.9)
Denmark	Falster	DK-201	11.32	54.92	Forest, high NDVI	1/60	1	0.2 (0.0–0.9)
Denmark	Jutland	DK-485	9.36	55.93	Forest, low NDVI	1/60	1	0.2 (0.0–0.9)
Denmark	Jutland	DK-521	10.68	56.26	Forest, low NDVI	1/60	1	0.2 (0.0–0.9)
Denmark	Jutland	DK-554	9.65	56.04	Forest, low NDVI	1/60	1	0.2 (0.0–0.9)
Denmark	Jutland	DK-604	9.27	56.3	Forest, high NDVI	1/60	1	0.2 (0.0–0.9)
Denmark	Jutland	DK-616	9.33	56.08	Forest, high NDVI	1/60	1	0.2 (0.0–0.9)
Sweden	Skåne	SE-005	14.44	56.45	Forest, low NDVI	3/60	3	0.5 (0.10–1.5)
Sweden	Skåne	SE-011	14.26	56.43	Forest, low NDVI	2/60	2	0.3 (0.04–1.2)
Sweden	Jonkoping	SE-064	14.99	57.2	Forest, low NDVI	1/60	1	0.2 (0.0–0.9)
Sweden	Kalmar	SE-122	15.61	56.72	Forest, high NDVI	1/60	1	0.2 (0.0–0.9)
Sweden	Karlskrona	SE-221	15.64	56.45	Forest, low NDVI	2/44	1, 1 lost[b]	0.2 (0.0–0.9)
Denmark	Central Zealand	DK-237	11.81	55.51	Forest, high NDVI	2/60	2 lost[b]	-
Denmark	Western Funen	DK-122	9.98	55.22	Forest, high NDVI	2/60	2 lost[b]	-
Denmark	North Jutland	DK-721	8.74	57.09	Meadow, low NDVI	1/60	1 lost[b]	-

[a] Pyrosequencing.

[b] TBEV RT-PCR-positive pools that were lost due to failure in pyrosequencing and could not be confirmed.

relationship with minimum SD. There was also a statistically significant positive correlation between EPP and mean temperature ($df=4$, $r=0.89$ and $p=0.02$) and minimum SD ($df=4$, $r=0.98$ and $p=0.00$) in summer (Table S2). In Denmark, we did not see any clear pattern. However, during the winter of 2015/2016, we observed a weak negative correlation between EPP and mean RH ($df=46$, $r=-0.33$, $p=0.02$) and a weak positive correlation between EPP and mean SD ($df=46$, $r=0.41$, $p=0.00$). There was also a weak positive correlation between EPP and mean SD ($df=34$, $r=0.366$, $p=0.03$) in the spring of 2016 (Table S3).

4 | DISCUSSION

This is the first study of TBEV in Scandinavia covering the whole Øresund-Kattegat-Skagerrak (ØKS) region. TBE is an emerging zoonosis in Scandinavia, which constitute the northernmost part of the distribution range of *I. ricinus* (Riccardi et al., 2019; Süss, 2011). Colder and dry winters and limited vegetation periods are a limiting factor for sustainable tick populations (van Oort et al., 2020) and thus the presence of TBEV in most of this region. The possible expansion of ticks into new regions in Scandinavia has been documented in earlier studies (Hvidsten et al., 2020; Lindquist & Vapalahti, 2008; Randolph, 2001). This study reports the presence of TBEV in tick populations in most parts of Denmark, which to our knowledge is reported for the first time. According to our analyses, TBEV prevalence in ticks in Denmark is widespread as we found 12 confirmed TBEV-positive sites, seven sites were in Jutland, three on Zealand and one each on Lolland and Bornholm (Figure 1, Table 2). Our PCR results suggest TBEV presence at several other sites, but these could not be confirmed due to technical issues (Table 2).

It appears that TBEV circulation in Denmark is not a recent development. Roe deer (*C. capreolus*) serum from 2003 to 2005 was analysed for the prevalence of tick-borne encephalitis complex virus and positives were found from southern Jutland, Lolland, Falster, northern Zealand and Bornholm (Skarphédinsson et al., 2005). The researchers indicated that although the TBE complex virus had emerged in new areas, the infection was still rare and focal in its distribution (Skarphédinsson et al., 2005). A follow-up study on the same deer species, roe deer sampled in 2013–2014 found that TBEV seropositive deers were found all over Denmark with a national seropositivity of 6.9%. Compared with the results from the study conducted in 2005, the seropositive roe deers had expanded to most areas of northern and central Jutland and Funen (Andersen, Larsen, et al., 2019; Skarphédinsson et al., 2005). The sentinel study also reported the presence of roe deer sera with high antibody titers from northern and central Jutland, which shows that the infection response in the deer could be a recent infection or it can be triggered by bites from multiple positive ticks. Our results confirm the findings that TBEV is more widespread in Denmark than previously anticipated. This study found seven positive sites from Jutland and supports TBEV foci being prevalent in Jutland. A seropositive roe deer was found on Funen (Andersen, Larsen, et al., 2019). In the present study, one PCR-positive pool was detected from Funen. Although the PCR-positive pool could not be confirmed positive due to technical difficulties during sequencing, this indicates that further assessment is needed to identify TBEV foci. The island of Bornholm in the Baltic Sea is a well-known TBEV endemic area since the 1950s (Kristiansen, 2002). The phylogenetic analysis of TBEV strains isolated from TBEV-infected patients from Bornholm showed relatedness to the eastern and central European strains indicating the expansion of TBEV infection from around the Baltics (Andersen, Bestehorn, et al., 2019). Besides Bornholm, human TBE cases have been reported from Tokkekøb Hegn and Tisvilde Hegn in northern Zealand, where the infecting TBEV strain clustered with the Norwegian strain, Mandal 2009 indicating possible expansion of TBEV from southern Norway or vice versa (Agergaard et al., 2019; Fomsgaard et al., 2013).

The previous roe deer and tick studies combined with the present study show that TBEV is circulating in Denmark and is more widespread than previously anticipated, although human TBE cases are rare. The low amount of human TBE cases could potentially be explained by TBEV not being a notifiable disease in Denmark. However, in Norway, TBEV is also prevalent in ticks and cervids (*C. capreolus*, *Cervus elaphus*, *Alces alces*) along the coast until 65°N (Paulsen et al., 2020; Soleng et al., 2018; Vikse et al., 2020), but human cases occur only in restricted foci along the southern coast (Andreassen et al., 2012; MSIS, 2022). Jutland and the western coast of Norway are both influenced by the North Sea, and it might be important to understand climatic influences on ticks and TBEV that can have special relevance to climate-sensitive zoonoses in the future. It is possible that a virus strain causing less severe disease circulates in these areas (Paulsen et al., 2015; Soleng et al., 2018; Vikse et al., 2020). The awareness of the general practitioners on the presence of TBEV might also play a role when diagnosing milder cases. TBEV detection methods also play a role in the detection of TBEV from questing ticks as the virus concentration is low (Schwaiger & Cassinotti, 2003). The length of the target sequence in the PCR might also be important to consider when the virus concentration is low in samples (Andreassen et al., 2012; Schwaiger & Cassinotti, 2003). False negatives due to limitations in the detection methods might be a reason for the lack of coherence in TBEV prevalence studies in ticks and sentinel studies in Denmark and Norway.

The present study found several new sites with relatively high TBEV prevalence along the Oslofjord in Norway. The Oslo and Viken counties in Norway are heavily populated areas where people drive to the city for work and live away from city areas. One location 45 km south of Oslo centrum showed EPP of 0.7% and another location near Sarpsborg, showed EPP of 0.5%. Both these locations are new locations where ticks were not screened for TBEV before. In the last few years, the incidence of TBE has been reported from new areas in Viken County (MSIS, 2022). As mentioned earlier, TBEV is endemic on the southern coast of Norway and there is a concern about the virus establishing in ticks in the more northeastern parts where Oslo is located. TBEV EPP of 0.2%–0.4% has been reported in Viken County from the former counties of Østfold, Akershus and Vestfold (Vikse et al., 2020) and the present study reports higher TBEV EPP along the Oslofjord in Norway than previously detected.

Although the presence of TBEV endemic foci along the western and southeastern coast was reported long ago, TBE foci in the inland part of southern Sweden is still a new emergence area (Brinkley et al., 2008; Fält et al., 2006; Lundkvist et al., 2011; Melik et al., 2007; Pettersson et al., 2014; Stjernberg et al., 2008; Waldeck et al., 2022). The nine sites in this study are from the seven neighbouring counties; Skåne, Kronoberg, Halland, Jönköping, Blekinge, Kalmar and Västragötaland (Figure 1). We found two positive sites from the northern part of Skåne County with five of eight positive pools resulting in TBEV EPP of 0.5% (Table 1). Several human TBE cases have also been reported in this area (Fält et al., 2006). A TBEV prevalence above 0.5% in questing ticks for more than 1 or 2 years has been used to define an endemic focus (Andreassen et al., 2012; Gäumann et al., 2010; Pettersson et al., 2014). Endemic foci refer to a geographical location in space and time where TBEV circulation is persistent in nature (Dobler et al., 2011). In other words, this area may be a risk area for possible TBEV infections in the future.

The positive sites in Blekinge and Kalmar counties in Sweden are close to the southeastern coast, where migrating birds may have introduced TBEV to the areas. Öland Island along the coast of Kalmar County, is a well-known bird migration stop (Waldenström et al., 2007) and it could potentially be a hotspot for the dispersal of TBEV-infected ticks by birds. TBEV isolated from a patient in Kalmar in 1993 has been whole genome sequenced and showed the closest resemblance to the NL/UH strain, isolated from *I. ricinus* ticks in the Netherlands and the Ljubljana strain, isolated from a patient in Slovenia (Paulsen et al., 2021). This suggests that there is a relationship between the long transport of ticks with TBEV infection and tick/human infection.

A potential study limitation is the single sampling between August 15th and September 30th, possibly missing peak infection rates in ticks due to differences in feeding cycles and how it aligns with seasonal spikes in reported cases. We might not capture peak infection rates within the ticks since these differences in detection rates may be based on feeding cycles in April to July versus August to November. However, according to the Norwegian Surveillance

System for Communicable Diseases (MSIS), TBE cases in Norway starts to appear in May, but the peak season is typically in August and September. This makes the timing of our sampling particularly fitting, as it aligns with the peak of TBE incidence.

We did not find any geographical clustering of TBEV in southern Scandinavia, unlike other tick-borne pathogens (Kjær et al., 2020). This could be attributed to a unique transmission potential of TBEV during cofeeding. Cofeeding transmission is identified as one of the major routes of TBEV transmission and maintenance in foci despite the low prevalence in tick population (Nah & Wu, 2021; Randolph et al., 2000). The formation of TBEV foci is a result of the complex interaction between ticks, their host species and the environment and hence might not exhibit a specific pattern.

Relative humidity is a crucial factor in deciding tick survival as ticks are very sensitive to desiccation, which might also affect the virus in the tick; however, very little is known about the effect of RH in TBEV prevalence in ticks (Danielová, 1990; Danielová et al., 1983; Korenberg, 2009; Sirotkin & Korenberg, 2019). This study suggests that humidity may play a major role in influencing TBEV prevalence in ticks in southern Scandinavia. In the northern part of Skåne in Sweden, TBEV prevalence in ticks was positively correlated with minimum humidity levels in all seasons. This suggests that higher air humidity may support more TBEV-positive ticks in areas with a continental climate like Sweden. TBEV virus was negatively correlated with maximum humidity and positively correlated with minimum dryness in coastal regions of southeastern Norway. This suggests that in regions where humidity levels are already high, lower humidity levels may be favourable. Overall, the study suggests that humidity might play an important role in TBEV virus prevalence; however, the effect may vary according to local microclimatic conditions. Further studies on understanding the influence of climatic parameters particularly the RH might be of special relevance under changing climatic conditions in Scandinavia. It is important to note that the obtained climatic parameters were from the nearest meteorological stations and provide a general relationship. The impact of the difference in scales in climate and microclimate has been discussed as ticks mostly live close to the ground where microclimatic conditions are modified by vegetation (Estrada-Peña et al., 2004; Randolph & Storey, 1999). It is important to consider that the relationship observed is based on few positive sites from Norway and Sweden and a larger number of study locations might give more information. It may be necessary for future studies to consider environmental data from local microclimate measurements rather than an aggregation of data collected from the national meteorological stations covering larger areas. This study confirms that TBEV is circulating in many locations throughout southern Scandinavia, pointing out that people acquiring tick bites in these areas are at risk of developing TBE and as such implying a public health concern. Future studies should aim to assess the impacts of climate change and monitor TBE foci in the region.

AUTHOR CONTRIBUTIONS

All authors revised and approved the final manuscript.

ACKNOWLEDGEMENTS

The ticks were collected as a part of the Scandinavian projects Interreg VA ScandTick Innovation Project (Project ID 20200422) and Capacity Building Project on Vector-borne Pathogens (The Norwegian Ministry of Health and Care Service (MoH) project B1710). We thank Dr. Preben Ottesen for his contribution to the tick collection and Ramesh Uprety for his help in acquiring climatic data.

FUNDING INFORMATION

This study was funded by ScandTick, Grant/Award Number: 167226; ScandTick Innovation, Grant/Award Number: 20200422 supported by the EU grant Interreg IVB and VA, ØKS program. ScandTick grant-award number, the Norwegian Ministry of Health and Care Services, Barents region project B1710 'Emerging infections: Capacity building on vector-borne infections in the Barents region, Norway and Russia'.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Ethical approval is not required for collecting and analysing tick samples in Norway.

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REFERENCES

Achazi, K., Růžek, D., Donoso-Mantke, O., Schlegel, M., Ali, H. S., Wenk, M., Schmidt-Chanasit, J., Ohlmeyer, L., Rühe, F., Vor, T., Kiffner, C., Kallies, R., Ulrich, R. G., & Niedrig, M. (2011). Rodents as sentinels for the prevalence of tick-borne encephalitis virus. *Vector Borne and Zoonotic Diseases*, *11*(6), 641–647.

Agergaard, C. N., Rosenstierne, M. W., Bødker, R., Rasmussen, M., Andersen, P. H. S., & Fomsgaard, A. (2019). New tick-borne encephalitis virus hot spot in Northern Zealand, Denmark, October 2019. *Eurosurveillance*, *24*(43), 1900639. https://doi.org/10.2807/1560-7917.ES.2019.24.43.1900639

Andersen, N. S., Bestehorn, M., Chitimia-Dobler, L., Kolmos, H. J., Jensen, P. M., Dobler, G., & Skarphédinsson, S. (2019). Phylogenetic characterization of tick-borne encephalitis virus from Bornholm, Denmark. *Ticks and Tick-Borne Diseases*, *10*(3), 533–539. https://doi.org/10.1016/j.ttbdis.2018.12.008

Andersen, N. S., Larsen, S. L., Olesen, C. R., Stiasny, K., Kolmos, H. J., Jensen, P. M., & Skarphédinsson, S. (2019). Continued expansion of tick-borne pathogens: Tick-borne encephalitis virus complex and *Anaplasma phagocytophilum* in Denmark. *Ticks and Tick-Borne Diseases*, *10*(1), 115–123. https://doi.org/10.1016/j.ttbdis.2018.09.007

Andreassen, A., Jore, S., Cuber, P., Dudman, S., Tengs, T., Isaksen, K., Hygen, H. O., Viljugrein, H., Anestad, G., Ottesen, P., &

Vainio, K. (2012). Prevalence of tick borne encephalitis virus in tick nymphs in relation to climatic factors on the southern coast of Norway. *Parasites & Vectors*, *5*(1), 177. https://doi.org/10.1186/1756-3305-5-177

Belova, O. A., Burenkova, L. A., & Karganova, G. G. (2012). Different tick-borne encephalitis virus (TBEV) prevalences in unfed versus partially engorged ixodid ticks – Evidence of virus replication and changes in tick behavior. *Ticks and Tick-Borne Diseases*, *3*(4), 240–246. https://doi.org/10.1016/j.ttbdis.2012.05.005

Brinkley, C., Nolskog, P., Golovljova, I., Lundkvist, Å., & Bergström, T. (2008). Tick-borne encephalitis virus natural foci emerge in western Sweden. *International Journal of Medical Microbiology*, *298*, 73–80.

Caini, S., Szomor, K., Ferenczi, E., Székelyné Gáspár, Á., Csohán, Á., Krisztalovics, K., Molnar, Z., & Horváth, J. K. (2012). Tick-borne encephalitis transmitted by unpasteurised cow milk in western Hungary, September to October 2011. *Eurosurveillance*, *17*(12), 20128. https://doi.org/10.2807/ese.17.12.20128-en

Cisak, E., Wójcik-Fatla, A., Zając, V., Sroka, J., Buczek, A., & Dutkiewicz, J. (2010). Prevalence of Tick-borne encephalitis virus (TBEV) in samples of raw milk taken randomly from cows, goats and sheep in eastern Poland. *Annals of Agricultural and Environmental Medicine*, *17*, 283–286.

Dai, X., Shang, G., Lu, S., Yang, J., & Xu, J. (2018). A new subtype of eastern tick-borne encephalitis virus discovered in Qinghai-Tibet Plateau, China. *Emerging Microbes & Infections*, *7*(1), 1–9. https://doi.org/10.1038/s41426-018-0081-6

Daniel, M., Danielová, V., Fialová, A., Malý, M., Kříž, B., & Nuttall, P. A. (2018). Increased relative risk of tick-borne encephalitis in warmer weather. *Frontiers in Cellular and Infection Microbiology*, *8*, 90. https://doi.org/10.3389/fcimb.2018.00090

Danielová, V. (1990). Experimental infection of ticks *Ixodes ricinus* with tick-borne encephalitis virus under. Different microclimatic conditions. *Folia Parasitologica*, *37*, 279–282.

Danielová, V., Daniel, M., Holubova, J., Hajkova, Z., Albrecht, V., Marhoul, Z., & Simonova, V. (1983). Influence of microclimatic factors on the development and virus infection rate of ticks *Ixodes ricinus* (L.) under experimental conditions. *Folia Parasitologica (Praha)*, *30*, 153–161.

Dobler, G., Hufert, F., Pfeffer, M., & Essbauer, S. (2011). Tick-borne encephalitis: From microfocus to human disease. In H. Mehlhorn (Ed.), *Progress in parasitology* (pp. 323–331). Springer.

Estrada-Peña, A., Martinez, J. M., Sanchez Acedo, C., Quilez, J., & Del Cacho, E. (2004). Phenology of the tick, *Ixodes ricinus*, in its southern distribution range (central Spain). *Medical and Veterinary Entomology*, *18*(4), 387–397. https://doi.org/10.1111/j.0269-283X.2004.00523.x

Fält, J., Lundgren, Å., Alsterlund, R., Carlsson, B., Eliasson, I., Haglund, M., Lundkvist, A., Vene, S., & Settergren, B. (2006). Tick-borne encephalitis (TBE) in Skåne, southern Sweden: A new TBE endemic region? *Scandinavian Journal of Infectious Diseases*, *38*(9), 800–804. https://doi.org/10.1080/00365540600664068

Fomsgaard, A. (2020) TBE in Denmark. Chapter 12b. *The TBE Book* (3rd ed.). Global Health Press. https://doi.org/10.33442/26613980_TBE-3_198

Fomsgaard, A., Christiansen, C. B., & Bødker, R. (2009). First identification of tick-borne encephalitis in Denmark outside of Bornholm, August 2009. *Eurosurveillance*, *14*(36), 19325. https://doi.org/10.2807/ese.14.36.19325-en

Fomsgaard, A., Fertner, M. E., Essbauer, S., Nielsen, A. Y., Frey, S., Lindblom, P., Lindgren, P. E., Bødker, R., Weidmann, M., & Dobler, G. (2013). Tick-borne encephalitis virus, Zealand, Denmark, 2011. *Emerging Infectious Diseases*, *19*(7), 1171–1173.

Gäumann, R., Mühlemann, K., Strasser, M., & Beuret, C. M. (2010). High-throughput procedure for tick surveys of tick-borne encephalitis virus and its application in a national surveillance study in Switzerland. *Applied and Environmental Microbiology*, *76*(13), 4241–4249. https://doi.org/10.1128/AEM.00391-10

Gritsun, T. S., Frolova, T. V., Zhankov, A. I., Armesto, M., Turner, S. L., Frolova, M. P., Pogodina, V. V., Lashkevich, V. A., & Gould, E. A. (2003). Characterization of a Siberian virus isolated from a patient with progressive chronic tick-borne encephalitis. *Journal of Virology*, *77*(1), 25–36. https://doi.org/10.1128/JVI.77.1.25-36.2003

Han, J., Zhu, L., Kulldorff, M., Hostovich, S., Stinchcomb, D. G., Tatalovich, Z., Lewis, D. R., & Feuer, E. J. (2016). Using Gini coefficient to determining optimal cluster reporting sizes for spatial scan statistics. *International Journal of Health Geographics*, *15*(1), 27. https://doi.org/10.1186/s12942-016-0056-6

Hanssen-Bauer, I., Førland, E., Haddeland, I., Hisdal, H., Lawrence, D., Mayer, S., Nesje, A., Nilsen, J. E., Sandven, S., Sandø, A., Sorteberg, A., & Ådlandsvik, B. (2017). Climate in Norway 2100.

Hillyard, P. D. (1996). *Ticks of north-west Europe*. Field Studies Council.

Hudopisk, N., Korva, M., Janet, E., Simetinger, M., Grgič-Vitek, M., Gubenšek, J., Natek, V., Kraigher, A., Strle, F., & Avšič-Županc, T. (2013). Tick-borne encephalitis associated with consumption of raw goat milk, Slovenia, 2012. *Emerging Infectious Diseases*, *19*(5), 806–808. https://doi.org/10.3201/eid1905.121442

Hvidsten, D., Frafjord, K., Gray, J. S., Henningsson, A. J., Jenkins, A., Kristiansen, B. E., Lager, M., Rognerud, B., Slåtsve, A. M., Stordal, F., Stuen, S., & Wilhelmsson, P. (2020). The distribution limit of the common tick, *Ixodes ricinus*, and some associated pathogens in north-western Europe. *Ticks and Tick-Borne Diseases*, *11*(4), 101388.

Jaenson, G. T., Petersson, E. H., Jaenson, D. G. E., Kindberg, J., Pettersson, J. H.-O., Hjertqvist, M., Medlock, J. M., & Bengtsson, H. (2018). The importance of wildlife in the ecology and epidemiology of the TBE virus in Sweden: Incidence of human TBE correlates with abundance of deer and hares. *Parasites & Vectors*, *11*(1), 477. https://doi.org/10.1186/s13071-018-3057-4

Jaenson, T. G., Jaenson, D. G., Eisen, L., Petersson, E., & Lindgren, E. (2012). Changes in the geographical distribution and abundance of the tick *Ixodes ricinus* during the past 30 years in Sweden. *Parasites & Vectors*, *5*(1), 8. https://doi.org/10.1186/1756-3305-5-8

Jaenson, T. G. T., & Wilhelmsson, P. (2019). First records of tick-borne pathogens in populations of the taiga tick *Ixodes persulcatus* in Sweden. *Parasites & Vectors*, *12*(1), 559. https://doi.org/10.1186/s13071-019-3813-0

Jung Kjær, L., Soleng, A., Edgar, K. S., Lindstedt, H. E. H., Paulsen, K. M., Andreassen, Å. K., Korslund, L., Kjelland, V., Slettan, A., Stuen, S., Kjellander, P., Christensson, M., Teräväinen, M., Baum, A., Klitgaard, K., & Bødker, R. (2019). Predicting the spatial abundance of *Ixodes ricinus* ticks in southern Scandinavia using environmental and climatic data. *Scientific Reports*, *9*(1), 18144. https://doi.org/10.1038/s41598-019-54496-1

Kaiser, R. (1999). The clinical and epidemiological profile of tick-borne encephalitis in southern Germany 1994–98: A prospective study of 656 patients. *Brain*, *122*(11), 2067–2078.

Kazarina, A., Japiņa, K., Keišs, O., Salmane, I., Bandere, D., Capligina, V., & Ranka, R. (2015). Detection of tick-borne encephalitis virus in *I. ricinus* ticks collected from autumn migratory birds in Latvia. *Ticks and Tick-Borne Diseases*, *6*(2), 178–180.

Kjær, L. J., Klitgaard, K., Soleng, A., Edgar, K. S., Lindstedt, H. E. H., Paulsen, K. M., Andreassen, Å. K., Korslund, L., Kjelland, V., Slettan, A., Stuen, S., Kjellander, P., Christensson, M., Teräväinen, M., Baum, A., Jensen, L. M., & Bødker, R. (2020). Spatial patterns of pathogen prevalence in questing *Ixodes ricinus* nymphs in southern Scandinavia, 2016. *Scientific Reports*, *10*(1), 19376. https://doi.org/10.1038/s41598-020-76334-5

Kjær, L. J., Soleng, A., Edgar, K. S., Lindstedt, H. E. H., Paulsen, K. M., Andreassen, Å. K., Korslund, L., Kjelland, V., Slettan, A., Stuen, S., Kjellander, P., Christensson, M., Teräväinen, M., Baum, A., Klitgaard, K., & Bødker, R. (2019). Predicting and mapping human risk of exposure to *Ixodes ricinus* nymphs using climatic and environmental data, Denmark, Norway and Sweden, 2016. *Eurosurveillance*, *24*(9), 1800101. https://doi.org/10.2807/1560-7917.ES.2019.24.9.1800101

Kleinman, K. (2015). *rsatscan: Tools, classes, and methods for interfacing with SaTScan stand-alone software*. Retrieved from https://CRAN.R-project.org/package=rsatscan

Klitgaard, K., Højgaard, J., Isbrand, A., Madsen, J. J., Thorup, K., & Bødker, R. (2019). Screening for multiple tick-borne pathogens in *Ixodes ricinus* ticks from birds in Denmark during spring and autumn migration seasons. *Ticks and Tick-Borne Diseases*, *10*(3), 546–552. https://doi.org/10.1016/j.ttbdis.2019.01.007

Korenberg, E. (2009). Recent epidemiology of tick-borne encephalitis: An effect of climate change? *Advances in Virus Research*, *74*, 123–144.

Kovalev, S. Y., & Mukhacheva, T. A. (2017). Reconsidering the classification of tick-borne encephalitis virus within the Siberian subtype gives new insights into its evolutionary history. *Infection, Genetics and Evolution*, *55*, 159–165. https://doi.org/10.1016/j.meegid.2017.09.014

Kozuch, O., & Nosek, J. (1980). Experimental transmission of tick-borne encephalitis (TBE) virus by Haemophysalis concinna ticks, *Acta virologica*, *24*(5), 377.

Kristiansen, K. (2002). TBE in Denmark-in particular on Bornholm. *International Journal of Medical Microbiology*, *291*, 62–63.

Kulldorff, M. (2018). *SaTScanTM v9.6: Software for the spatial and space-time scan statistics*. Information Management Services, Inc. Retrieved from www.satscan.org

Larsen, A. L., Kanestrøm, A., Bjørland, M., Andreassen, Å., Soleng, A., Vene, S., & Dudman, S. G. (2014). Detection of specific IgG antibodies in blood donors and tick-borne encephalitis virus in ticks within a non-endemic area in southeast Norway. *Scandinavian Journal of Infectious Diseases*, *46*(3), 181–184. https://doi.org/10.3109/00365548.2013.865140

Lindquist, L., & Vapalahti, O. (2008). Tick-borne encephalitis. *The Lancet*, *371*(9627), 1861–1871. https://doi.org/10.1016/S0140-6736(08)60800-4

Lindström, A., & Jaenson, T. G. T. (2003). Distribution of the common tick, *Ixodes ricinus* (Acari: Ixodidae), in different vegetation types in southern Sweden. *Journal of Medical Entomology*, *40*(4), 375–378. https://doi.org/10.1603/0022-2585-40.4.375

Lundkvist, Å., Wallensten, A., Vene, S., & Hjertqvist, M. (2011). Tick-borne encephalitis increasing in Sweden, 2011. *Eurosurveillance*, *16*(39), 19981.

Marvik, Å., Tveten, Y., Pedersen, A.-B., Stiasny, K., Andreassen, Å. K., & Grude, N. (2021). Low prevalence of tick-borne encephalitis virus antibodies in Norwegian blood donors. *Infectious Diseases*, *53*(1), 44–51. https://doi.org/10.1080/23744235.2020.1819561

Medlock, J. M., Hansford, K. M., Bormane, A., Derdakova, M., Estrada-Peña, A., George, J.-C., Golovljova, I., Jaenson, T. G., Jensen, J. K., Jensen, P. M., Kazimirova, M., Oteo, J. A., Papa, A., Pfister, K., Plantard, O., Randolph, S. E., Rizzoli, A., Santos-Silva, M. M., Sprong, H., … van Bortel, W. (2013). Driving forces for changes in geographical distribution of *Ixodes ricinus* ticks in Europe. *Parasites & Vectors*, *6*(1), 1. https://doi.org/10.1186/1756-3305-6-1

Melik, W., Nilsson, A. S., & Johansson, M. (2007). Detection strategies of tick-borne encephalitis virus in Swedish *Ixodes ricinus* reveal evolutionary characteristics of emerging tick-borne flaviviruses. *Archives of Virology*, *152*(5), 1027–1034. https://doi.org/10.1007/s00705-006-0922-9

Michelitsch, A., Wernike, K., Klaus, C., Dobler, G., & Beer, M. (2019). Exploring the reservoir hosts of tick-borne encephalitis virus. *Viruses*, *11*(7), 669. https://doi.org/10.3390/v11070669

MSIS. (2022). *Skogflåttencefalitt (TBE-virusinfeksjoner (2022)). Norwegian surveillance system for communicable diseases*. Retrieved from https://www.fhi.no/nettpub/smittevernveilederen/sykdommer-a-a/skogflattencefalitt-tbe-virusinfeks

Nah, K., & Wu, J. (2021). Long-term transmission dynamics of tick-borne diseases involving seasonal variation and co-feeding transmission. *Journal of Biological Dynamics*, *15*(1), 269–286.

Paulsen, K. M., das Neves, C., Granquist, E. G., Madslien, K., Stuen, S., Pedersen, B. N., Vikse, R., Rocchi, M., Laming, E., Stiasny, K., & Andreassen, Å. K. (2020). Cervids as sentinel-species for tick-borne encephalitis virus in Norway—A serological study. *Zoonoses and Public Health*, *67*(4), 342–351. https://doi.org/10.1111/zph.12675

Paulsen, K. M., Lamsal, A., Bastakoti, S., Pettersson, J. H.-O., Pedersen, B. N., Stiasny, K., Haglund, M., Smura, T., Vapalahti, O., Vikse, R., Alfsnes, K., & Andreassen, Å. K. (2021). High-throughput sequencing of two European strains of tick-borne encephalitis virus (TBEV), Hochosterwitz and 1993/783. *Ticks and Tick-Borne Diseases*, *12*(1), 101557. https://doi.org/10.1016/j.ttbdis.2020.101557

Paulsen, K. M., Pedersen, B. N., Soleng, A., Okbaldet, Y. B., Pettersson, J. H.-O., Dudman, S. G., Ottesen, P., Vik, I. S., Vainio, K., & Andreassen, Å. (2015). Prevalence of tick-borne encephalitis virus in *Ixodes ricinus* ticks from three islands in north-western Norway. *APMIS*, *123*(9), 759–764. https://doi.org/10.1111/apm.12412

Paulsen, K. M., Stuen, S., das Neves, C. G., Suhel, F., Gurung, D., Soleng, A., Stiasny, K., Vikse, R., Andreassen, Å. K., & Granquist, E. G. (2019). Tick-borne encephalitis virus in cows and unpasteurized cow milk from Norway. *Zoonoses and Public Health*, *66*(2), 216–222. https://doi.org/10.1111/zph.12554

Perret, J.-L., Rais, O., & Gern, L. (2004). Influence of climate on the proportion of *Ixodes ricinus* nymphs and adults questing in a tick population. *Journal of Medical Entomology*, *41*(3), 361–365. https://doi.org/10.1603/0022-2585-41.3.361

Pettersson, J. H.-O., Golovljova, I., Vene, S., & Jaenson, T. G. (2014). Prevalence of tick-borne encephalitis virus in *Ixodes ricinus* ticks in northern Europe with particular reference to Southern Sweden. *Parasites & Vectors*, *7*(1), 102. https://doi.org/10.1186/1756-3305-7-102

Public Health Agency of Sweden. (2021). *Tick-borne encephalitis (TBE)-disease statistics 2016–2021*. Retrieved December 18, 2022, from https://www.folkhalsomyndigheten.se/folkhalsorapportering-statistik/statistik-a-o/sjukdomsstatistik/tick-borne-encephalitis-tbe/?t=county

R Development Core Team. (2018). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing. https://www.R-project.org/

Randolph, S., Gern, L., & Nuttall, P. (1996). Co-feeding ticks: Epidemiological significance for tick-borne pathogen transmission. *Parasitology Today*, *12*(12), 472–479.

Randolph, S. E. (2001). The shifting landscape of tick-borne zoonoses: Tick-borne encephalitis and Lyme borreliosis in Europe. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *356*(1411), 1045–1056. https://doi.org/10.1098/rstb.2001.0893

Randolph, S. E. (2011). Transmission of tick-borne pathogens between co-feeding ticks: Milan Labuda's enduring paradigm. *Ticks and Tick-Borne Diseases*, *2*(4), 179–182. https://doi.org/10.1016/j.ttbdis.2011.07.004

Randolph, S. E., Green, R. M., Peacey, M. F., & Rogers, D. J. (2000). Seasonal synchrony: The key to tick-borne encephalitis foci identified by satellite data. *Parasitology*, *121*(1), 15–23. https://doi.org/10.1017/S0031182099006083

Randolph, S. E., & Storey, K. (1999). Impact of microclimate on immature tick-rodent host interactions (Acari: Ixodidae): Implications for parasite transmission. *Journal of Medical Entomology*, *36*(6), 741–748.

Riccardi, N., Antonello, R. M., Luzzati, R., Zajkowska, J., Di Bella, S., & Giacobbe, D. R. (2019). Tick-borne encephalitis in Europe: A brief update on epidemiology, diagnosis, prevention, and treatment. *European Journal of Internal Medicine*, *62*, 1–6.

Schwaiger, M., & Cassinotti, P. (2003). Development of a quantitative real-time RT-PCR assay with internal control for the laboratory detection of tick borne encephalitis virus (TBEV) RNA. *Journal of Clinical Virology*, *27*(2), 136–145. https://doi.org/10.1016/S1386-6532(02)00168-3

Sirotkin, M. B., & Korenberg, E. I. (2019). Influence of abiotic factors on infectious agents environmentally associated with Ixodidae ticks on the example of borrelia and tick-borne encephalitis virus. *Biology Bulletin Reviews*, *9*(6), 543–561. https://doi.org/10.1134/S2079086419060070

Skarphédinsson, S., Jensen, P. M., & Kristiansen, K. (2005). Survey of tickborne infections in Denmark. *Emerging Infectious Diseases*, *11*(7), 1055–1061. https://doi.org/10.3201/eid1107.041265

Slunge, D., Boman, A., & Studahl, M. (2022). Burden of tick-borne encephalitis, Sweden. *Emerging Infectious Diseases*, *28*(2), 314–322. https://doi.org/10.3201/eid2802.204324

Soleng, A., Edgar, K. S., Paulsen, K. M., Pedersen, B. N., Okbaldet, Y. B., Skjetne, I. E. B., Gurung, D., Vikse, R., & Andreassen, Å. K. (2018). Distribution of *Ixodes ricinus* ticks and prevalence of tick-borne encephalitis virus among questing ticks in the Arctic Circle region of northern Norway. *Ticks and Tick-Borne Diseases*, *9*(1), 97–103. https://doi.org/10.1016/j.ttbdis.2017.10.002

Statens Serum Institut. (2020). *TBE report on disease occurrence 2017–2019*. Statens Serum Institut. Retrieved from https://en.ssi.dk/surveillance-and-preparedness/surveillance-in-denmark/annual-reports-on-disease-incidence/tbe-report-on-disease-occurrence-2017-2019

Stjernberg, L., Holmkvist, K., & Berglund, J. (2008). A newly detected tick-borne encephalitis (TBE) focus in south-east Sweden: A follow-up study of TBE virus (TBEV) seroprevalence. *Scandinavian Journal of Infectious Diseases*, *40*(1), 4–10. https://doi.org/10.1080/00365540701522934

Süss, J. (2011). Tick-borne encephalitis 2010: Epidemiology, risk areas, and virus strains in Europe and Asia—An overview. *Ticks and Tick-Borne Diseases*, *2*(1), 2–15.

Svensson, J., Christiansen, C. B., & Persson, K. E. M. (2021). A serosurvey of tick-borne encephalitis virus in Sweden: Different populations and geographical locations. *Vector Borne and Zoonotic Diseases*, *21*(8), 614–619. https://doi.org/10.1089/vbz.2020.2763

Thortveit, E. T., Aase, A., Petersen, L. B., Lorentzen, Å. R., Mygland, Å., & Ljøstad, U. (2020). Human seroprevalence of antibodies to tick-borne microbes in southern Norway. *Ticks and Tick-Borne Diseases*, *11*(4), 101410. https://doi.org/10.1016/j.ttbdis.2020.101410

van Oort, B. E. H., Hovelsrud, G. K., Risvoll, C., Mohr, C. W., & Jore, S. (2020). A mini-review of *Ixodes* ticks climate sensitive infection dispersion risk in the Nordic Region. *International Journal of Environmental Research and Public Health*, *17*(15), 5387. https://doi.org/10.3390/ijerph17155387

Vikse, R., Paulsen, K. M., Edgar, K. S., H-O Pettersson, J., Ottesen, P., Okbaldet, Y. B., Kiran, N., Lamsal, A., Lindstedt, H. E. H., Pedersen, B. N., Soleng, A., & Andreassen, Å. K. (2020). Geographical distribution and prevalence of tick-borne encephalitis virus in questing *Ixodes ricinus* ticks and phylogeographic structure of the *Ixodes ricinus* vector in Norway. *Zoonoses and Public Health*, *67*(4), 370–381. https://doi.org/10.1111/zph.12696

Waldeck, M., Winqvist, N., Henriksson, G., Dyrdak, R., Settergren, B., & Lindgren, P.-E. (2022). Surveillance of tick-borne encephalitis in emerging risk areas in southern Sweden: A retrospective case finding study. *European Journal of Clinical Microbiology & Infectious Diseases*, *42*, 13–22. https://doi.org/10.1007/s10096-022-04509-1

Waldenström, J., Lundkvist, Å., Falk, K. I., Garpmo, U., Bergström, S., Lindegren, G., Sjöstedt, A., Mejlon, H., Fransson, T., Haemig, P. D., & Olsen, B. (2007). Migrating birds and tickborne encephalitis virus. *Emerging Infectious Diseases*, *13*(8), 1215–1218. https://doi.org/10.3201/eid1308.061416

Wallenhammar, A., Lindqvist, R., Asghar, N., Gunaltay, S., Fredlund, H., Davidsson, Å., Andersson, S., Överby, A. K., & Johansson, M. (2020). Revealing new tick-borne encephalitis virus foci by screening antibodies in sheep milk. *Parasites & Vectors*, *13*(1), 185. https://doi.org/10.1186/s13071-020-04030-4

Wilhelmsson, P., Jaenson, T. G. T., Olsen, B., Waldenström, J., & Lindgren, P.-E. (2020). Migratory birds as disseminators of ticks and the tick-borne pathogens Borrelia bacteria and tick-borne encephalitis (TBE) virus: A seasonal study at Ottenby Bird Observatory in South-eastern Sweden. *Parasites & Vectors*, *13*(1), 607. https://doi.org/10.1186/s13071-020-04493-5

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Lamsal, A., Edgar, K. S., Jenkins, A., Renssen, H., Kjær, L. J., Alfsnes, K., Bastakoti, S., Dieseth, M., Klitgaard, K., Lindstedt, H. E. H., Paulsen, K. M., Vikse, R., Korslund, L., Kjelland, V., Stuen, S., Kjellander, P., Christensson, M., Teräväinen, M., Jensen, L. M. … Andreassen, Å. K. (2023). Prevalence of tick-borne encephalitis virus in questing *Ixodes ricinus* nymphs in southern Scandinavia and the possible influence of meteorological factors. *Zoonoses and Public Health*, *00*, 1–12. https://doi.org/10.1111/zph.13049

Paper II

Lamsal, A., Tryland, M., Paulsen, K. M., Romano, J. S., Nymo, I. H., Stiasny, K., Soleng, A., Vikse, R., & Andreassen, Å. K. (2023). Serological screening for tick-borne encephalitis virus in eight Norwegian herds of semi-domesticated reindeer (*Rangifer tarandus tarandus*). *Zoonoses and Public Health*, 70, 692–698. https://doi.org/10.1111/zph.13060

Received: 7 September 2022 | Revised: 11 May 2023 | Accepted: 14 May 2023
DOI: 10.1111/zph.13060

SHORT COMMUNICATION

WILEY

Serological screening for tick-borne encephalitis virus in eight Norwegian herds of semi-domesticated reindeer (*Rangifer tarandus tarandus*)

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Funding information
Fram Center flagship "Effects of climate change on terrestrial ecosystems, landscapes, society and indigenous peoples", Grant/Award Number: 362256; Norwegian Ministry of Health and Care Services, Barents region project B1710 "Emerging infections: Capacity building on vector-borne infections in the Barents region, Norway and Russia"; Norwegian Reindeer Development

Abstract

Tick-borne encephalitis virus (TBEV) is found in *Ixodes ricinus* ticks throughout the area where viable tick populations exist. In Norway, TBEV is found in *I. ricinus* from the south coast until Brønnøy municipality in Nordland County and the range of the vector is expanding due to changes in climate, vegetation, host animals and environmental conditions. TBEV might thus have the potential to establish in new areas when *I. ricinus* expand its geographical distribution. At present, there is little knowledge on the status of the virus in high-altitude areas of inland regions in Norway. It has previously been indicated that reindeer may be an important sentinel species and indicator of the spread of ticks and TBEV in high-altitude regions. In this study, 408 semi-domesticated Eurasian tundra reindeer (*Rangifer tarandus tarandus*) from eight herds, from Tana in Troms and Finnmark County in northern Norway to Filefjell in Innlandet and Viken Counties in southern Norway, were screened for TBEV antibodies using a commercial enzyme-linked immunosorbent assay (ELISA). We found 16 TBEV reactive reindeer samples by ELISA; however, these results could not be confirmed by the serum neutralization test (SNT). This could indicate that a flavivirusand not necessarily TBEV, may be circulating among Norwegian semi-domesticated reindeer. The results also indicate that TBEV was not enzootic in Norwegian semi-domesticated

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reindeer in 2013–2015. This knowledge is important as an information base for future TBEV and flavivirus surveillance in Norway.

KEYWORDS
climate change, flavivirus, *Ixodes ricinus*, sentinels, serology, TBEV, ticks

1 | INTRODUCTION

The burden of vector-borne flaviviruses like tick-borne encephalitis virus (TBEV), West Nile virus, Usutu virus, as well as other vector-borne viruses like picornaviruses, Sindbis virus and Inkoo virus, is increasing in northern Europe (Lim et al., 2018; Shakya et al., 2022; Slunge et al., 2022; Tingström et al., 2016). Ticks and mosquitoes, along with their avian and mammalian hosts, facilitate the enzootic maintenance of these viruses in nature (Esser et al., 2019). The arctic and subarctic regions are facing impacts of climate change faster than the global average (Box et al., 2019). Climate change is upsurging favourable conditions for circulation of these viruses in nature by modifying microclimatic conditions, thus facilitating changes in vegetation and in the population density and geographical distribution of animal hosts. All together, these changes increase the risk of zoonotic infections (Chala & Hamde, 2021; Esser et al., 2019). There is, however, little knowledge on the geographical spread of these flaviviruses in colder arctic regions.

TBEV is a medically important flavivirus that causes encephalitis in humans and animals with possible fatal neurological symptoms (Kaiser, 2002). The severity of the disease is broad, ranging from asymptomatic, to mild fever and headache, and to meningitis or meningoencephalitis (Lindquist & Vapalahti, 2008). Five subtypes of TBEV have been identified: European (TBEV-Eu), Siberian (TBEV-Sib), Far Eastern subtype (TBEV-Fe), Baikalian (TBEV-Bkl) and Himalayan (TBEV-Him) (Dai et al., 2018; Kovalev & Mukhacheva, 2017). The European subtype is present in the coastal areas of Norway with *Ixodes ricinus* ticks as the main vectors (Andreassen et al., 2012; Paulsen et al., 2015; Soleng et al., 2018; Vikse et al., 2020).

Although infected ticks are reported as far north as Brønnøy Municipality in Nordland County, human incidence is limited to the south. This may be related to underdiagnosis or circulation of a milder strain in the northern region (Vikse et al., 2020). In Norway, TBEV foci are identified by the presence of infected cervids such as moose (*Alces alces*), red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*) (Paulsen et al., 2020; Ytrehus et al., 2013). These cervids are hence important sentinels to identify TBEV foci in particular areas, and the results can thereafter be coupled with screening of ticks and other animals (Balling et al., 2014; Paulsen et al., 2020; Skarphédinsson et al., 2005; Ytrehus et al., 2013).

Reindeer is a key cervid species with broad social and ecological value in Norway and are mostly herded by indigenous Sami herders (Riseth et al., 2019). Reindeer are adapted to colder climates than other deer species, having a wider distribution from the coast to higher altitudes, both in the Taiga and Tundra areas (Nowak, 1999). Reindeer have been documented to support enzootic maintenance of viruses like herpesvirus, pestivirus and Hepatitis E virus (Rinaldo et al., 2021; Romano et al., 2021; Sacristán et al., 2021; Tryland et al., 2021). Recently, antibodies against Inkoo virus and Inkoo virus-specific RNA were detected in reindeer for the first time in Norway which indicates that reindeer are exposed to a wide range of viruses (Shakya et al., 2022). The aim of the present study was to use semi-domesticated reindeer from eight regions as sentinels to evaluate TBEV distribution from northern to southern Norway.

Impacts

- This study contributes to a better understanding of the TBEV-status in areas of Norway where ticks are present, but not abundant.
- This study supports previous findings for TBEV in ticks in Norway, that is, that TBEV is present in coastal areas and not in inland areas.
- We recommended further research to identify potential flaviviruses circulating in reindeer in the future.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Serum samples ($n = 480$) from semi-domesticated reindeer were collected (Tryland et al., 2021) from eight different reindeer herding regions: Tana, Lakselv, Tromsø, Lødingen, Hattfjelldal, Fosen, Røros and Filefjell during the winter seasons (October–April) of 2013, 2014 and 2015 (Table 1, Figure 1). The serum samples were stored at −80°C until further analysis.

2.2 | Serological methods

The serum samples were screened for TBEV immunoglobulin G (IgG) antibodies by a modified commercial enzyme-linked immunosorbent assay (ELISA), Enzygnost® Anti-TBE virus IgG, (Siemens) at The Norwegian Institute of Public Health, Oslo. The ELISA method was

TABLE 1 Seroprevalence of tick-borne encephalitis virus or a similar flavivirus by ELISA in Eurasian tundra reindeer (*Rangifer tarandus tarandus*) from eight different herding districts in.

		TBEV positive (borderline)/total tested			
4County	**Location**	**2013**	**2014**	**2015**	**Total**
Troms and Finnmark	Tana	0 (0)/20	0 (0)/20	2 (0)/20	2 (0)/60
Troms and Finnmark	Lakselv	0 (1)/20	0 (1)/20	0 (1)/20	0 (3)/60
Troms and Finnmark	Tromsø	0 (0)/21	0 (1)/20	0 (0)/20	0 (1)/61
Nordland	Lødingen	0 (0)/22	2 (1)/20	–	2 (1)/42
Nordland	Hattfjelldal	0 (0)/30	0 (0)/20	0 (1)/20	0 (1)/70
Trøndelag	Fosen	0 (0)/20	0 (0)/20	0 (0)/20	0 (0)/60
Trøndelag	Røros	0 (1)/22	1 (1)/20	2 (1)/20	3 (3)/62
Innlandet and Viken	Filefjell	0 (0)/25	0 (0)/20	0 (0)/20	0 (0)/65
	Total	0 (2)/180	3 (4)/160	4 (3)/140	7 (9)/480

FIGURE 1 Map of Norway illustrating reindeer sampling locations. Map created using the Free and Open Source QGIS. Map data: © OpenStreetMap-Mitwirkende, SRTM | Map position: © OpenTopoMap 129 (CC-BY-SA). Reindeer pastures data source: NIBIO.

modified using peroxidase-labelled affinity purified antibody to deer IgG (H+L) in rabbit (TriChem ApS-interkemi) and adapted for deer samples as described previously (Paulsen et al., 2020). Previously confirmed TBE IgG positive and negative moose and roe deer serum samples by SNT were used as internal controls (Paulsen et al., 2020). Positive TBE ELISA results were re-tested by a TBEV-specific SNT at the Medical University of Vienna, Centre for Virology (Austria) as described previously (Malafa et al., 2020; Stiasny et al., 2009). The virus neutralization titre was defined as the reciprocal of the serum dilution that gave 90% reduction in the absorbance readout compared with the control without antibody. An SNT titre ≥10 was considered positive.

3 | RESULTS AND DISCUSSION

A total of 229 adult and 251 calf («calves of the year») reindeer serum samples, from eight different reindeer herding districts distributed geographically from north to south of Norway, were screened for antibodies against TBEV using ELISA and SNT. Seven serum samples (1.5%) were classified as positive and nine (1.9%) as borderline for TBEV by the ELISA (Table 1). The reindeer classified as positive were sampled in Tana ($n=2$; 2015), Lødingen ($n=2$; 2014) and Røros ($n=1$; 2014 and $n=2$; 2015) (Table 1). Reindeer classified as borderline were detected in the other herding districts, except for Fosen and Filefjell. None of the samples were positive by the SNT (Table S1); hence, the TBEV infection in reindeer could not be confirmed.

The lack of coherence between the ELISA and the SNT in this study might be due to serological cross-reactions among similar flaviviruses based on close antigenic similarity (Krzysiak et al., 2021; Lim et al., 2018; Marvik et al., 2021; Paulsen et al., 2020). A previous study also showed that TBEV ELISA reactive reindeer were negative in the SNT (Paulsen et al., 2020). Thus, these ELISA results are probably reflecting an exposure of reindeer to a virus closely related to TBEV, which could be tick-borne or mosquito-borne in origin. Louping ill virus (LIV), a tick-borne flavivirus closely related to TBEV, has been found in sheep in Norway in the 1980s, and antibodies against LIV have been found in willow ptarmigan (*Lagopus lagopus lagopus*) and deer species like red deer, but not in reindeer (Paulsen et al., 2020; Ulvund, 1987; Ytrehus et al., 2013). Mosquito-borne flaviviruses like West Nile virus (WNV) and Lammi virus (LAMV) show serological cross reactivity with TBEV (Huhtamo et al., 2009; Tonteri et al., 2016). Although WNV have been found to infect deer and cause clinical disease, this is probably rare (Palmer et al., 2004; Tonteri et al., 2016). In the study by Tonteri et al. (2016), 1371 cervids (moose, white-tailed deer and roe deer) were tested for TBEV in Finland, of which 10 were positive for TBEV and two of these were positive for LAMV. In addition, two TBEV negative were positive for LAMV. None of the deer were infected by WNV (Tonteri et al., 2016). Based on these findings, LAMV is shown to circulate in deer species. This could be due to cross reaction or double infection LAMV is detected in mosquitoes from Finland (Huhtamo et al., 2009) and as the cervids are known to be exposed to both tick and mosquito vectors, Tonteri et al. (2016) deemed it necessary to include the examination of both WNV and LAMV in their study. Notably, a subset of the deer samples tested were seropositive for LAMV and TBEV (Tonteri et al., 2016). In our study, the positive TBEV ELISA results that were negative in SNT (Table 1) may suggest reactivity with other flaviviruses as a result of exposure to mosquito bites.

Previous studies have shown that an unidentified flavivirus closely related to TBEV and LIV, may be circulating far north along the western coast of Norway (Ytrehus et al., 2013). Unlike ticks, mosquitoes are found all over Norway, including the inland regions (Shakya et al., 2022). Sequencing of RNA from reindeer nasal and/or rectal swabs have identified viral sequences representing the *Flaviviridae* family in reindeer in Norway, which is hosted mainly by arthropod vectors like ticks and mosquitoes (Romano et al., 2021). Furthermore, the mosquito-borne virus INKV was detected in mosquitoes collected from Røros and INKV-specific antibodies were detected in reindeer from all the reindeer herding areas included in this study (Shakya et al., 2022). In the present study, the TBE ELISA-positive samples came from six out of eight locations from north to south of Norway where viable mosquito populations have been reported by Sánchez Romano et al. (2021) and Shakya et al. (2022) (Figure 1, Table 1). Most of the positive samples came from Røros in Trøndelag County, followed by Lødingen in Nordland County and Lakselv in Troms and Finnmark County (Table 1). These findings support that reindeer in these areas are exposed to mosquito-bites. Therefore, we report that Norwegian semi-domesticated reindeer are in some areas, exposed to a TBEV-related flavivirus, that might be a mosquito-borne flavivirus (Table 1). This highlights the need for additional study to determine the prevalence of LAMV and other flavivirus infections among mosquitoes and cervids in Norway.

Reindeer herding covers large geographic areas, typically in mountain tundra regions of Norway, with long winter migration to pastures in inland regions (Riseth et al., 2019; Tryland et al., 2021). However, in a few areas, like in Nordland County, reindeer herds are more oriented to the coastal regions using snow free areas for pasture during the winter (Riseth et al., 2019). The inland regions are dryer, colder and generally geographically elevated compared with the coastal regions (Hanssen-Bauer et al., 2017), and hence sustainable tick populations cannot thrive (De Pelsmaeker et al., 2021; Qviller et al., 2014). Therefore, TBEV infection in tick populations is reported mostly from coastal regions of Norway (Andreassen et al., 2012; Soleng et al., 2018; Vikse et al., 2020). TBEV seropositive moose, roe deer and red deer have been detected in the southern and western parts of Norway (Paulsen et al., 2020). Reindeer, have, however, to our knowledge, never been reported seropositive for TBEV, which might be due to absence of TBEV

TABLE 2 Distribution by gender and age of reindeer classified as Positive and Borderline for anti-TBEV antibodies using a commercial ELISA (Enzygnost® Anti-TBE virus IgG).

	Number of reactive samples for TBEV ELISA test	Borderline	Positive
Adult female	7/189	3	4
Calf[a] female	5/102	3	2
Adult male	0/40	0	0
Calf[a] male	2/135	2	0
Unknown	2/14	1	1
Total	16/480	9	7

[a] Calf of the year (born in April–May, sampled in October–April).

infected ticks in the reindeer pasture areas. However, climate change is expected to modify existing environmental conditions in northern and inland regions of Norway in the future (Hanssen-Bauer et al., 2017). This might promote circulation of flaviviruses in new environments by supporting life cycles of arthropod hosts such as ticks and mosquitoes. Therefore, surveillance of flavivirus circulation should be continued and should include key indicator species such as reindeer.

The seroprevalence for TBEV was higher in female reindeer (4.1%) compared with males (1.1%) (Table 2). Similar to this study, a higher number of INKV-seropositive female reindeer were reported in an earlier study, based on the same sample collection (Shakya et al., 2022). The sample collection is, however, biased, due to a restricted availability of bulls during the sampling period. Thus, only 40 adult males were included compared with 189 adult females, which may have had impact on the results.

This study is an important baseline study for future research involving reindeer and ticks in Norway. General knowledge on climate change effects and adaptation strategies has increased significantly in recent years, but there is still a substantial information gap regarding the influence of climate change on zoonotic diseases. Changes in climate may give opportunities for vectors and flaviviruses to occur in new areas of Norway. It is, therefore, important to continue the surveillance of both vectors and viruses.

AUTHOR CONTRIBUTIONS

MT, JSR and IHN collected reindeer sera samples, AL did ELISA analysis, interpretation and drafted the manuscript. ÅKA designed and initiated the study, supervision, data analysis, interpretation, critically revising the manuscript and proofreading; RV and AS contributed by supervision, writing, revising the manuscript and proofreading and KMP contributed to ELISA assay, revising and proofreading the manuscript. KS performed the SNT analysis and revised the manuscript draft. All authors revised and approved the final manuscript.

ACKNOWLEDGEMENTS

Thanks to all the staff at Department of Virology, Norwegian Institute of Public Health, for technical support. We thank reindeer herders for their patience and assistance during the collection of reindeer serum samples.

FUNDING INFORMATION

This study was funded by the Norwegian Ministry of Health and Care Services, Barents region project B1710 'Emerging infections: Capacity building on vector-borne infections in the Barents region, Norway and Russia'. The sample collection was funded by the Norwegian Reindeer Development Fund and by the Fram Center flagship 'Effects of climate change on terrestrial ecosystems, landscapes, society and indigenous peoples' grant no. 362256.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Sampling was conducted in cooperation with reindeer herders as a general health surveillance when animals were gathered and handled for other purposes, and the study was not classified as an animal experiment. Informed consent was obtained from the herd owners for the participation of their animals in this study.

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REFERENCES

Andreassen, Å. K., Jore, S., Cuber, P., Dudman, S. G., Tengs, T., Isaksen, K., Hygen, H. O., Viljugrein, H., Ånestad, G., Ottesen, P., & Vainio, M. K. (2012). Prevalence of tick borne encephalitis virus in tick nymphs in relation to climatic factors on the southern coast of Norway. *Parasites & Vectors*, *5*(1), 177. https://doi.org/10.1186/1756-3305-5-177

Balling, A., Plessow, U., Beer, M., & Pfeffer, M. (2014). Prevalence of antibodies against tick-borne encephalitis virus in wild game from Saxony, Germany. *Ticks and Tick-Borne Diseases*, *5*(6), 805–809. https://doi.org/10.1016/j.ttbdis.2014.06.007

Box, J. E., Colgan, W. T., Christensen, T. R., Schmidt, N. M., Lund, M., Parmentier, F.-J. W., Brown, R., Bhatt, U. S., Euskirchen, E. S., Romanovsky, V. E., Walsh, J. E., Overland, J. E., Wang, M., Corell, R. W., Meier, W. N., Wouters, B., Mernild, S., Mård, J., Pawlak, J., & Olsen, M. S. (2019). Key indicators of Arctic climate change: 1971–2017. *Environmental Research Letters*, *14*(4), 045010. https://doi.org/10.1088/1748-9326/aafc1b

Chala, B., & Hamde, F. (2021). Emerging and Re-emerging vector-borne infectious diseases and the challenges for control: A review. *Frontiers in Public Health*, *9*, 715759. https://doi.org/10.3389/fpubh.2021.715759

Dai, X., Shang, G., Lu, S., Yang, J., & Xu, J. (2018). A new subtype of eastern tick-borne encephalitis virus discovered in Qinghai-Tibet plateau, China. *Emerging Microbes & Infections*, *7*(1), 1–9. https://doi.org/10.1038/s41426-018-0081-6

De Pelsmaeker, N., Korslund, L., & Steifetten, Ø. (2021). High-elevational occurrence of two tick species, Ixodes ricinus and *I. trianguliceps*, at their northern distribution range. *Parasites & Vectors*, *14*(1), 161. https://doi.org/10.1186/s13071-021-04604-w

Esser, H. J., Mögling, R., Cleton, N. B., van der Jeugd, H. P., Sprong, H., Stroo, A., Koopmans, M., de Boer, W. F., & Reusken, C. (2019). Risk factors associated with sustained circulation of six zoonotic arboviruses: A systematic review for selection of surveillance sites in non-endemic areas. *Parasites & Vectors*, *12*(1), 265. https://doi.org/10.1186/s13071-019-3515-7

Hanssen-Bauer, I., Førland, E., Haddeland, I., Hisdal, H., Lawrence, D., Mayer, S., Nesje, A., Nilsen, J. E., Sandven, S., Sandø, A., Sorteberg, A., & Ådlandsvik, B. (2017). *Climate in Norway 2100*. Norwegian Environment Agency. https://www.miljodirektoratet.no/M741

Huhtamo, E., Putkuri, N., Kurkela, S., Manni, T., Vaheri, A., Vapalahti, O., & Uzcátegui, N. Y. (2009). Characterization of a novel flavivirus from mosquitoes in northern Europe that is related to mosquito-borne flaviviruses of the tropics. *Journal of Virology*, *83*(18), 9532–9540. https://doi.org/10.1128/JVI.00529-09

Kaiser, R. (2002). Tick-borne encephalitis (TBE) in Germany and clinical course of the disease. *International Journal of Medical Microbiology*, *291*, 58–61. https://doi.org/10.1016/S1438-4221(02)80012-1

Kovalev, S. Y., & Mukhacheva, T. A. (2017). Reconsidering the classification of tick-borne encephalitis virus within the Siberian subtype gives new insights into its evolutionary history. *Infection, Genetics and Evolution*, *55*, 159–165. https://doi.org/10.1016/j.meegid.2017.09.014

Krzysiak, M. K., Anusz, K., Konieczny, A., Rola, J., Salat, J., Salat, J., Straková, P., Olech, W., & Larska, M. (2021). The European bison (*Bison bonasus*) as an indicatory species for the circulation of tick-borne encephalitis virus (TBEV) in natural foci in Poland. *Ticks and Tick-Borne Diseases*, *12*(6), 101799. https://doi.org/10.1016/j.ttbdis.2021.101799

Lim, S. M., Geervliet, M., Verhagen, J. H., Müskens, G. J. D. M., Majoor, F. A., Osterhaus, A. D. M. E., & Martina, B. E. E. (2018). Serologic evidence of West Nile virus and Usutu virus infections in Eurasian coots in the Netherlands. *Zoonoses and Public Health*, *65*(1), 96–102. https://doi.org/10.1111/zph.12375

Lindquist, L., & Vapalahti, O. (2008). Tick-borne encephalitis. *The Lancet*, *371*(9627), 1861–1871. https://doi.org/10.1016/S0140-6736(08)60800-4

Malafa, S., Medits, I., Aberle, J. H., Aberle, S. W., Haslwanter, D., Tsouchnikas, G., Wölfel, S., Huber, K. L., Percivalle, E., Cherpillod, P., Thaler, M., Roßbacher, L., Kundi, M., Heinz, F. X., & Stiasny, K. (2020). Impact of flavivirus vaccine-induced immunity on primary zika virus antibody response in humans. *PLoS Neglected Tropical Diseases*, *14*(2), e0008034. https://doi.org/10.1371/journal.pntd.0008034

Marvik, Å., Tveten, Y., Pedersen, A.-B., Stiasny, K., Andreassen, Å. K., & Grude, N. (2021). Low prevalence of tick-borne encephalitis virus antibodies in Norwegian blood donors. *Infectious Diseases*, *53*(1), 44–51. https://doi.org/10.1080/23744235.2020.1819561

Nowak, R. (1999). Walker's mammals of the world. In (6th ed., Vol. 2, pp. 1128–1130): Johns Hopkins University Press.

Palmer, M. V., Stoffregen, W. C., Rogers, D. G., Hamir, A. N., Richt, J. A., Pedersen, D. D., & Waters, W. R. (2004). West Nile virus infection in reindeer (*Rangifer tarandus*). *Journal of Veterinary Diagnostic Investigation*, *16*(3), 219–222. https://doi.org/10.1177/104063870401600307

Paulsen, K. M., das Neves, C. G., Granquist, E. G., Madslien, K., Stuen, S., Pedersen, B. N., Vikse, R., Rocchi, M., Rocchi, M., Laming, E., Laming, E., Stiasny, K., & Andreassen, Å. K. (2020). Cervids as sentinel-species for tick-borne encephalitis virus in Norway—A serological study. *Zoonoses and Public Health*, *67*(4), 342–351. https://doi.org/10.1111/zph.12675

Paulsen, K. M., Pedersen, B. N., Pedersen, B. N., Soleng, A., Okbaldet, Y. B., Pettersson, J. H.-O., Dudman, S. G., Ottesen, P., Vik, I. S. S., Vainio, K., & Andreassen, Å. K. (2015). Prevalence of tick-borne encephalitis virus in *Ixodes ricinus* ticks from three islands in North-Western Norway. *APMIS*, *123*(9), 759–764. https://doi.org/10.1111/apm.12412

Qviller, L., Grøva, L., Viljugrein, H., Klingen, I., & Mysterud, A. (2014). Temporal pattern of questing tick *Ixodes ricinus* density at differing elevations in the coastal region of western Norway. *Parasites & Vectors*, *7*(1), 179. https://doi.org/10.1186/1756-3305-7-179

Rinaldo, C. H., Nymo, I. H., Sánchez Romano, J., Breines, E. M., Murguzur, F. J. A., & Tryland, M. (2021). Serological evidence of hepatitis E virus infection in semi-domesticated Eurasian tundra reindeer (*Rangifer tarandus tarandus*) in Norway. *Pathogens*, *10*(12), 1542. https://doi.org/10.3390/pathogens10121542

Riseth, J., Tømmervik, H., & Forbes, B. (2019). Ch.1.3 Sustainable and resilient reindeer herding. In M. Tryland & S. J. Kutz (Eds.), *Reindeer and caribou: Health and disease* (pp. 23–43). CRC Press. https://www.researchgate.net/profile/Hans-Tommervik/publication/344787755_Ch13_Sustainable_and_resilient_reindeer_herding/links/5f9066a592851c14bcd8c74e/Ch13-Sustainable-and-resilient-reindeer-herding.pdf

Romano, J. S., Omazic, A., Leijon, M., Hagström, Å., Tryland, M., Kantanen, J., Reilas, T., Rockström, U., Fedorov, V., & Albihn, A. (2021). Screening of Eurasian tundra reindeer for viral sequences by next-generation sequencing. *International Journal of Environmental Research and Public Health*, *18*(12), 6561. https://doi.org/10.3390/ijerph18126561

Sacristán, C., Madslien, K., Sacristán, I., Klevar, S., & das Neves, C. G. (2021). Seroprevalence of hepatitis E virus in moose (*Alces alces*), reindeer (*Rangifer tarandus*), Red Deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), and muskoxen (*Ovibos moschatus*) from Norway. *Viruses*, *13*(2), 224. https://doi.org/10.3390/v13020224

Shakya, R., Tryland, M., Vikse, R., Romano, J. S., Åsbakk, K., Nymo, I. H., Mehl, R., Evander, M., Ahlm, C., Vapalahti, O., Lwande, O. W., Putkuri, N., Johansen, W., Soleng, A., Edgar, K. S., & Andreassen, Å. K. (2022). Inkoo and Sindbis viruses in blood sucking insects, and a serological study for Inkoo virus in semi-domesticated Eurasian tundra reindeer in Norway. *Virology Journal*, *19*(1), 99. https://doi.org/10.1186/s12985-022-01815-0

Skarphédinsson, S., Jensen, P. M., & Kristiansen, K. (2005). Survey of tickborne infections in Denmark. *Emerging Infectious Diseases*, *11*(7), 1055–1061. https://doi.org/10.3201/eid1107.041265

Slunge, D., Boman, A., & Studahl, M. (2022). Burden of tick-borne encephalitis, Sweden. *Emerging Infectious Diseases*, *28*(2), 314–322. https://doi.org/10.3201/eid2802.204324

Soleng, A., Edgar, K. S., Paulsen, K. M., Pedersen, B. N., Pedersen, B. N., Okbaldet, Y. B., Skjetne, I. E. B., Gurung, D., Vikse, R., & Andreassen, Å. K. (2018). Distribution of *Ixodes ricinus* ticks and prevalence of tick-borne encephalitis virus among questing ticks in the Arctic circle region of northern Norway. *Ticks and Tick-Borne Diseases*, *9*(1), 97–103. https://doi.org/10.1016/j.ttbdis.2017.10.002

Stiasny, K., Holzmann, H., & Heinz, F. X. (2009). Characteristics of antibody responses in tick-borne encephalitis vaccination breakthroughs. *Vaccine*, *27*(50), 7021–7026. https://doi.org/10.1016/j.vaccine.2009.09.069

Tingström, O., Wesula Lwande, O., Näslund, J., Spyckerelle, I., Engdahl, C., Von Schoenberg, P., Ahlm, C., Evander, M., & Bucht, G. (2016). Detection of Sindbis and Inkoo virus RNA in genetically typed mosquito larvae sampled in northern Sweden. *Vector-Borne and Zoonotic Diseases*, *16*(7), 461–467. https://doi.org/10.1089/vbz.2016.1940

Tonteri, E., Jokelainen, P., Matala, J., Pusenius, J., & Vapalahti, O. (2016). Serological evidence of tick-borne encephalitis virus infection in moose and deer in Finland: Sentinels for virus circulation. *Parasites & Vectors*, *9*(1), 54. https://doi.org/10.1186/s13071-016-1335-6

Tryland, M., Romano, J. S., Nymo, I. H., Breines, E. M., Murguzur, F. J. A., Murguzur, F. J. A., Kjenstad, O. C., Li, H., & Cunha, C. W. (2021). A screening for virus infections in eight herds of semi-domesticated Eurasian tundra reindeer (*Rangifer tarandus tarandus*) in Norway, 2013–2018. *Frontiers in Veterinary Science*, *8*, 707787. https://doi.org/10.3389/fvets.2021.707787

Ulvund, M. (1987). Louping-ill (tick-borne encephalitis) hos sau i vest-Norge [Louping-ill (tick-borne encephalitis) in sheep in western Norway]. *Smafenytt*, *7*, 99–106.

Vikse, R., Paulsen, K. M., Edgar, K. S., Pettersson, J. H.-O., Ottesen, P., Okbaldet, Y. B., Kiran, N., Lamsal, A., Lindstedt, H. E. H., Pedersen, B. N., Soleng, A., & Andreassen, Å. K. (2020). Geographical distribution and prevalence of tick-borne encephalitis virus in questing *Ixodes ricinus* ticks and phylogeographic structure of the *Ixodes* *ricinus* vector in Norway. *Zoonoses and Public Health*, *67*(4), 370–381. https://doi.org/10.1111/zph.12696

Ytrehus, B., Vainio, K., Dudman, S. G., Gilray, J., & Willoughby, K. (2013). Tick-borne encephalitis virus and louping-ill virus may co-circulate in southern Norway. *Vector-Borne and Zoonotic Diseases*, *13*(10), 762–768. https://doi.org/10.1089/vbz.2012.1023

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Lamsal, A., Tryland, M., Paulsen, K. M., Romano, J. S., Nymo, I. H., Stiasny, K., Soleng, A., Vikse, R., & Andreassen, Å. K. (2023). Serological screening for tick-borne encephalitis virus in eight Norwegian herds of semi-domesticated reindeer (*Rangifer tarandus tarandus*). *Zoonoses and Public Health*, *00*, 1–7. https://doi.org/10.1111/zph.13060

Paper III

Lamsal, A., Diekmann, MJ., Paulsen, E.Q., Dorenberg, D.C.H., Růžek, D., Salat, J., Vikse, R., Soleng, A., & Andreassen, Å. K. A retrospective study on the prevalence of TBEV-specific antibodies in patients with CNS symptoms admitted to Oslo University Hospital, Oslo Norway. *Manuscript draft*.

Not available online

Paper IV

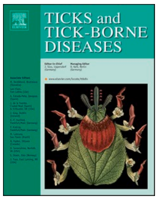
Paulsen, KM., Lamsal, A., Bastakoti, S., Pettersson, J. H., Pedersen, B.N., Stiasny, K., Haglund, M., Smura, T., Vapalahti, O., Vikse, R., Alfsnes, K., & Andreassen, Å. K. High-throughput sequencing of two European strains of tick-borne encephalitis virus (TBEV), Hochosterwitz and 1993/783. Ticks and Tick Borne Diseases, 2021 Jan;12(1):101557. doi: 10.1016/j.ttbdis.2020.101557.

Ticks and Tick-borne Diseases 12 (2021) 101557

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis



Short communication

High-throughput sequencing of two European strains of tick-borne encephalitis virus (TBEV), Hochosterwitz and 1993/783

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ARTICLE INFO

Keywords:
TBEV
Flaviviruses
WGS
Phylogeny
Ambiguous nucleotide

ABSTRACT

Tick-borne encephalitis virus (TBEV) is a medically important arbovirus, widespread in Europe and Asia. The virus is primarily transmitted to humans and animals by bites from ticks and, in rare cases, by consumption of unpasteurized dairy products. The aim of this study was to sequence and characterize two TBEV strains with amplicon sequencing by designing overlapping primers. The amplicon sequencing, via Illumina MiSeq, covering nearly the entire TBEV genome, was successful: We retrieved and characterized the complete polyprotein sequence of two TBEV strains, Hochosterwitz and 1993/783 from Austria and Sweden, respectively. In this study the previous phylogenetic analysis of both strains was confirmed to be of the European subtypes of TBEV (TBEV-Eu) by whole genome sequencing. The Hochosterwitz strain clustered with the two strains KrM 93 and KrM 213 from South Korea, and the 1993/783 strain clustered together with the NL/UH strain from the Netherlands. Our study confirms the suitability and rapidness of the high-throughput sequencing method used to produce complete TBEV genomes from TBEV samples of high viral load giving high-molecular-weight cDNA with large overlapping amplicons.

1. Introduction

Tick-borne encephalitis virus (TBEV) is a medically important arthropod-borne virus (arbovirus), which is widespread across large parts of Europe and Asia. TBEV is the causative agent of the disease tick-borne encephalitis (TBE) in humans and animals (Lindquist and Vapalahti, 2008; Suss, 2011). TBEV is mainly transmitted to humans and animals through bites from *Ixodes ricinus* and *Ixodes persulcatus* ticks, and in rare cases through ingestion of unpasteurized dairy products (Balogh et al., 2010; Brockmann et al., 2018; Holzmann et al., 2009; Hudopisk et al., 2013; Kerlik et al., 2018; Paulsen et al., 2019; Ruzek et al., 2019). Some small mammals are proven reservoirs for the TBEV, while migratory birds and large mammals are important for distribution of ticks and the virus (Carpi et al., 2008; Mlera and Bloom, 2018; Nuttall

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https://doi.org/10.1016/j.ttbdis.2020.101557
Received 6 April 2020; Received in revised form 28 August 2020; Accepted 1 September 2020
Available online 14 October 2020

and Labuda, 2003; Waldenström et al., 2007).

Taxonomically, TBEV belongs to the genus *Flavivirus* within the family *Flaviviridae*. The TBEV genome consist of approximately 11 kb positive-sense single-stranded RNA. The viral RNA encodes one single open reading frame (ORF) of about 3400 amino acids, flanked by 5′ and 3′ non-coding regions (NCRs), three structural proteins; envelope (E), precursor membrane (PrM) and capsid (C), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A-2K-4B complex and NS5) (Heinz and Mandl, 1993; Kaufusi et al., 2014; Plaszczyca et al., 2019; Ruzek et al., 2019; Slavik et al., 1970). Based on phylogenetic grouping and geographical distribution five subtypes of TBEV are known: the European, Siberian, Far Eastern, Baikalian and Himalayan subtypes (Adelshin et al., 2019; Dai et al., 2018; Ecker et al., 1999; Kovalev and Mukhacheva, 2017). These five subtypes are distributed over the Eurasian continent from Europe, Russia, Japan, China and South Korea (Demina et al., 2010; Dobler et al., 2012; Yoshii et al., 2017). The European TBEV subtype (TBEV-Eu) is prevalent across Europe and Asia, from England, France, the Netherlands, to east Sibiria in Russia and South Korea (Dekker et al., 2019; Demina et al., 2010; Dobler et al., 2012; Holding et al., 2019; Ruzek et al., 2019; Velay et al., 2018). Most of the available TBEV-Eu sequences originate from ticks. Conversely, few sequences are available from clinical TBE cases because they often are PCR-negative on the onset of neurological symptoms (Haglund et al., 2003; Saksida et al., 2005).

In this work we sequenced two cultured TBEV strains; one originating from a Swedish patient (1993/783) (Haglund et al., 2003) and one from an Austrian tick collected in 1971 (Hochosterwitz) (Heinz and Kunz, 1981). The aim of this study was to characterize the two TBEV strains. We established a high-throughput amplicon sequencing method with overlapping primers on Illumina MiSeq, based on a protocol developed by Quick et al. (2017).

2. Materials and methods

2.1. Virus strains and virus-cultivation

The Hochosterwitz strain was isolated from an *I. ricinus* tick collected in 1971 near the Hochosterwitz palace in Austria, which is considered a highly endemic TBE area. The strain 1993/783 originated from a patient hospitalized in 1993 at Kalmar Hospital, Sweden, with a moderate form of TBE with symptoms such as fever, malaise, myalgia and headache (Haglund et al., 2003). The Hochosterwitz strain had been passaged two times in mouse brain and then passaged several times in African green monkey kidney Vero E6 cells. Strain 1993/783 had initially been passaged once in suckling baby mice and once in Vero E6 cells. Prior to the experiments, Hochosterwitz were passaged eight times and 1993/783 three times in Vero E6 cell culture. The viral titre for Hochosterwitz was approximately 6.5×10^6 focus forming units according to the protocol by Stiasny et al. (2009) per ml, while the titre for 1993/783 was unknown (Stiasny et al., 2009).

2.2. RNA extraction and reverse transcription (RT) of viral RNA

Viral RNA was extracted from the cultivated virus stocks of the strains using QIAamp® Viral RNA mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's recommendations. Immediately after extraction, the viral RNA was reversely transcribed to cDNA using SuperScript III reverse transcription kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with random primers and RNase inhibitor (Applied Biosystems, Foster City, California, USA) according to the manufacturers' protocol.

2.3. Primer design, polymerase chain reaction and gel electrophoresis

To recover the complete coding TBEV genome, we followed the "Primal Scheme" as described in Quick et al. (2017). Briefly, 54 TBEV genomes representing the European TBEV genetic diversity were retrieved from NCBI GenBank and aligned using Muscle 3.8.425 (Edgar, 2004). Primers were designed using the online resource "Primal Scheme" (Quick et al., 2017), with amplicon length set to 2000 nt and overlap to 200 nt (Table 1). PCR was performed following the protocol with the Q5 high fidelity polymerase enzyme described in Quick et al. (2017).

2.4. Library preparation and high-throughput sequencing

The PCR products were cleaned using 1.8x Ampure XP beads (Beckman Coulter Life Sciences, Indianapolis, Indiana) according to the Kapa HyperPlus Kit clean-up protocol (KAPA Biosystems, Roche, Basel, Switzerland). Library preparation and amplification were performed using KAPA HyperPlus (KAPA Biosystems, Roche, Basel, Switzerland) and sequenced using a MiSeq using 2 × 300 v3 (Illumina, San Diego, California), following to the manufacturers' recommendations.

2.5. High-throughput sequence data processing and assembly

Sequencing reads from the separate products representing each strain were concatenated, reads smaller than 50 nt and poor quality reads were removed using Trim Galore v0.4.1 (a wrapper by Felix Krueger at the Babraham Institute using Cutadapt v1.18 (default quality trimming; $Q < 30$) (Martin, 2011) and FastQC (Simon Andrews also at the Babraham Institute)). Reads from each of the two strains were mapped to a library of TBEV sequences (Table S1) using BowTie2 v2.3.4.3 (with the local alignment option) (Langmead and Salzberg, 2012). Aligned reads were further processed using Samtools v1.9 (Li, 2011) and weeSAM v1.4 (Centre for Virus Research, Glasgow, UK). The highest number of reads from the sequenced Hochosterwitz strain mapped to KrM 93 (HM535611.1), and the 1993/783 strain to NL/UH (MH021184.1), consequently these two were used as references for a second round of reference-based assembly using the same software. Consensus sequences of Hochosterwitz and 1993/783 were called from

Table 1
Primer pairs used to sequence the two tick-borne encephalitis virus strains Hochosterwitz and 1993/783.

Primer name	Position*	Sequences (5' to 3')	Primer pair
JK_1_Forward	35-57	AGC ATT AGC AGC GGT TGG TTT G	Primer pair 1
JK_1_Reverse	1998-1976	GAC TGG GAT CCT ACA GGG CTT T	
JK_2_Forward	1742-1764	CGG AGA CCA GAC TGG AGT GTT A	Primer pair 2
JK_2_ Reverse	3770-3748	AAC ACA GCC TGG AGT AGC ATC A	
JK_3_ Forward	3511-3533	TTG CGG ACA ACG GTG AAT TAC T	Primer pair 3
JK_3_ Reverse	5343-5321	GAA CCT GAC CCG TTT CCC ATT C	
JK_4_ Forward	5077-5099	ATG AGA CCT ACG TCA GCA GCA T	Primer pair 4
JK_4_ Reverse	6918-6896	CAT CTC ATT GGC TGC AAC CAG T	
JK_5_Forward	6671-6693	CTT CGT CGT CCG GAC TTC AAT C	Primer pair 5
JK_5_ Reverse	8660-8638	GGC CAG CTG AGA AGT TTC ACA A	
JK_6_ Forward	8384-8406	ACT TTT GGC TCG GTT TGG AGA C	Primer pair 6
JK_6_ Reverse	10261-10239	CCC AGA TGT TCT TGG CCC ATT C	
JK_7_ Forward	9347-9369	GCA CAA ACA ATT GGC AAC CAC A	Primer pair 7
JK_7_ Reverse	11197-11175	ATT TCT CTC TTC CCT CCT CCC G	

* The positions correspond to the consensus alignment of 54 TBEV genomes, representing European TBEV genetic diversity, retrieved from NCBI GenBank.

the resulting alignment using the built-in consensus caller in Geneious Prime v2020.1, calling bases matching at least 50% of the reads, only reads mapping the region corresponding to the amplified products JK1–JK7 were considered (entire region had >1000 coverage).

2.6. Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignment was performed using Muscle 3.8.425 (Edgar, 2004) on genomes described in (Table S1) in addition to one strain from Denmark (Andersen et al., 2019), and 14 sequences from Finland (Smura et al., 2019).

Nucleotide model selection was performed in ModelFinder integrated in IQ-TREE 1.6.11 (Nguyen et al., 2015). The evolutionary history of the complete polyprotein alignment was inferred using the maximum likelihood method with the GTR + F+I + G4 model of nucleotide substitution using IQ-TREE 1.6.11. Branch support expressed as Shimodaira–Hasegawa approximate likelihood-ratio test (SH-aLTR) and ultrafast bootstrap were both calculated by computing 1000 replicates. The Louping ill virus (GenBank accession number: NC_001809.1, strain 369/T2) a closely related flavivirus, was chosen as an outgroup.

3. Results and discussion

The use of amplicon sequencing with overlapping primers has previously been documented as a successful approach to sequence the whole genome of clinical Zika virus samples (Quick et al., 2017). In this study, we designed seven TBEV-Eu primer pairs with fragments of approximately 2000 nt based on the protocol of Quick et al. (2017) and sequenced two TBEV-Eu strains: Hochosterwitz (GenBank accession number MT311861) and 1993/783 (GenBank accession number MT311860). Summary of the sequencing coverage and number of reads is given in Table S2. Since we were sequencing cultivated viruses with high viral load, we designed primers for amplification of longer fragments than Quick et al. (2017). The use of shorter amplicon length, such as 400 nt may be useful for samples with low viral load and/or degraded viral RNA. The amplicon approach allows for multiplexing of samples, and affordable sequencing of the low abundance virus RNA in tick and patient samples.

Both the Hochosterwitz and the 1993/783 sequences contained 10,871 nt, consisting of 76 nt 5’ NCR, 10,245 nt polyprotein, and 551 nt 3’ NCR. The poly(A) tract was mostly deleted (or truncated) but had retained the same pattern of (A)3C(A)6 in both strains. This is identical to the short poly(A) tract retained in the Toro 2003 and Habo 2011 strains. The role of the heterogenic poly(A) tracts in the life cycle of the virus is not clear. However, deep sequencing of Toro 2003 clones after passaging in cell culture or mouse brain revealed mutations in specific genomic regions, indicative of culture driven selection. In addition, mutations within the poly(A) tract are suggested to be an important virulence determinant for TBEV or related to virus cultivation. A longer sequence of the poly(A) tract seemed more common in virus cultivated in mice compared to cell culture (Asghar et al., 2016; Asghar et al., 2014; Mandl et al., 1991). Our sequence result of the NS5 region of 1993/783 showed 100% identity with a previously published sequence of the same region (GenBank accession number KF991109).

Phylogenetic analysis of the complete coding region of Hochosterwitz and 1993/783 confirmed that both strains belong to the TBEV-Eu subtype (Fig. 1). The Hochosterwitz strain grouped together with two endemic South Korean strains, KrM 93 and KrM 213, both belonging to the TBEV-Eu subtype (Yun et al., 2011). This is surprising due to the geographical distance from Europe, and the observation that the neighbouring countries Japan and China mainly harbour the far eastern (TBEV-Fe) subtype (Ko et al., 2010; Yoshii et al., 2017). Migratory birds may have introduced TBEV-Eu strains into South Korea (Carpi et al., 2008; Mlera and Bloom, 2018; Nuttall and Labuda, 2003; Waldenström et al., 2007). Tick populations with TBEV-Eu are found both in Western and Eastern Siberia of Russia (Demina et al., 2010). Considering the relatively short feeding time (five to nine days), it is possible that *Ixodes* ticks may have been acquired by migratory birds at stopovers or transported non-stop over this long-distance (Klaus et al., 2016). There is also a possibility of rodent reservoirs being involved in virus distribution through international sea trade.

The phylogenetic analysis demonstrated that the strain 1993/783 clustered with both the NL/UH strain from the Netherlands, and with the strains from Slovenia (Ljubljana) and Finland (Isosaari) (Fig. 1). The strain 1993/783 and the Slovenia (Ljubljana) strain both originated from human cases. The Swedish patient (1993/783) with a moderate

Fig. 1. Maximum likelihood tree showing the TBEV-Eu diversity. Node numbers represent bootstrap values (SH-aLRT support (%) / ultrafast bootstrap support (%)). Scale bar show number of nucleotide changes. Samples in this study show in red, TBEV-Eu reference strain Neudoerfl shown in blue.

TBE disease was presumably infected in the Kalmar municipality in Sweden (Haglund et al., 2003), while the strain from Slovenia (Ljubljana) originated from a severe human TBE case, most likely infected by aerosols while working with TBEV in the laboratory (Avsic-Zupanc et al., 1995).

The various European TBEV strains do generally not display any geographical clustering with regards to their host origin. Strains from the Netherlands, Sweden and Finland belong to different clusters though they all originated from ticks. The 1993/783 strain from the patient did not cluster with the other Nordic strains originating from ticks, like the Finnish (Sipoo and Espoo) or the Swedish strains (Saringe, Torö, JP-296 and JP-554) (Fig. 1). The overall genetic variation of TBEV does not seem to be host dependent since the strains isolated from patients cluster with the tick strains and vice versa (Fig. 1) (Grubaugh et al., 2019). We do not find any geographic clustering in this study, confirming previous studies suggesting a lack of a distinct phylogeographic pattern in TBEV-Eu strains (Heinze et al., 2012).

We identified 52 and eight ambiguous nucleotide positions in the sequence reads of the Hochosterwitz and 1993/783 strains, respectively (Table S3). One of the ambiguous nucleotides, at position 1923, were primer induced. Exchange of amino acids with different biochemical properties affecting polarity or charge due to different folding or protein function, might be critical for the survival, transmission and replication of TBEV. The main ambiguous nucleotides can be explained by either PCR, sequencing induced errors, passage history in laboratory mice and mammalian cell lines or indicate sub-populations or quasispecies (Grubaugh et al., 2019; Romanova et al., 2007). However, the same amplification methods were used for both TBEV strains and, if the minority populations detected here were due to polymerase or sequencing errors, one might expect similar rates in both TBEV strains (Potapov and Ong, 2017). It is difficult to explain the higher number of ambiguous sites in structural genes of Hochosterwitz strain compared to non-structural genes or 1993/783 strain by PCR-errors. We would expect that PCR-errors were more or less evenly distributed in the genome.

In summary, the amplicon sequencing of two TBEV-Eu strains was successful. We retrieved and characterized the complete polyprotein sequence of Hochosterwitz and 1993/783 from Austria and Sweden, respectively.

4. Conclusions

Our study confirms a method for high-throughput sequencing of TBEV samples of high viral load giving high-molecular-weight cDNA with large overlapping amplicons. This offers an improved tool for TBEV sequencing and diagnostics of TBE. The multiplex PCR protocol has advantages as it reduces the cost of reagents and minimises the possibility of laboratory errors. Studying virus populations within naturally infected humans and ticks can lead to breakthrough in our understanding of virus-host interactions and novel approaches for surveillance.

Funding

This study was supported the EU Interreg V-A program Scandtick Innovation project [grant number 20200422]; the Norwegian Ministry of Health and Care Services, Barents region program [grant number B1412 and B1710]; Swedish research council FORMAS [grant number 2015-710].

Declaration of Competing Interest

None

Acknowledgements

Sincere thanks to Sirkka Vene for guidance and cultivation of the TBEV strains, and to Professor Franz X. Heinz, Medical University of Vienna, Austria for kindly providing the Hochosterwitz strain and to Kedar Gautam for contribution to the laboratory work.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2020.101557.

References

Adelshin, R.V., Sidorova, E.A., Bondaryuk, A.N., Trukhina, A.G., Sherbakov, D.Y., White Iii, R.A., Andaev, E.I., Balakhonov, S.V., 2019. "886-84-like" tick-borne encephalitis virus strains: Intraspecific status elucidated by comparative genomics. Ticks Tick Borne Dis 10 (5), 1168–1172. https://doi.org/10.1016/j.ttbdis.2019.06.006.

Andersen, N.S., Larsen, S.L., Olesen, C.R., Stiasny, K., Kolmos, H.J., Jensen, P.M., Skarphedinsson, S., 2019. Continued expansion of tick-borne pathogens: Tick-borne encephalitis virus complex and *Anaplasma phagocytophilum* in Denmark. Ticks Tick Borne Dis 10 (1), 115–123. https://doi.org/10.1016/j.ttbdis.2018.09.007.

Asghar, N., Lee, Y.P., Nilsson, E., Lindqvist, R., Melik, W., Kroger, A., Overby, A.K., Johansson, M., 2016. The role of the poly(A) tract in the replication and virulence of tick-borne encephalitis virus. Sci Rep 6, 39265. https://doi.org/10.1038/srep39265.

Asghar, N., Lindblom, P., Melik, W., Lindqvist, R., Haglund, M., Forsberg, P., Overby, A. K., Andreassen, A., Lindgren, P.E., Johansson, M., 2014. Tick-borne encephalitis virus sequenced directly from questing and blood-feeding ticks reveals quasispecies variance. PLoS One 9 (7), e103264. https://doi.org/10.1371/journal.pone.0103264.

Avsic-Zupanc, T., Poljak, M., Maticic, M., Radsel-Medvescek, A., LeDuc, J.W., Stiasny, K., Kunz, C., Heinz, F.X., 1995. Laboratory acquired tick-borne meningoencephalitis: characterisation of virus strains. Clin Diagn Virol 4 (1), 51–59. Retrieved from. https://www.ncbi.nlm.nih.gov/pubmed/15566827.

Balogh, Z., Ferenczi, E., Szeles, K., Stefanoff, P., Gut, W., Szomor, K.N., Takacs, M., Berencsi, G., 2010. Tick-borne encephalitis outbreak in Hungary due to consumption of raw goat milk. J Virol Methods 163 (2), 481–485. https://doi.org/10.1016/j.jviromet.2009.10.003.

Brockmann, S., Oehme, R., Buckenmaier, T., Beer, M., Jeffery-Smith, A., Spannenkrebs, M., Haag-Milz, S., Wagner-Wiening, C., Schlegel, C., Fritz, J., Zange, S., Bestehorn, M., Lindau, A., Hoffmann, D., Tiberi, S., Mackenstedt, U., Dobler, G., 2018. A cluster of two human cases of tick-borne encephalitis (TBE) transmitted by unpasteurised goat milk and cheese in Germany, May 2016. Eurosurveillance 23 (15), 17–00336. https://doi.org/10.2807/1560-7917.ES.2018.23.15.17-00336.

Carpi, G., Cagnacci, F., Neteler, M., Rizzoli, A., 2008. Tick infestation on roe deer in relation to geographic and remotely sensed climatic variables in a tick-borne encephalitis endemic area. Epidemiol Infect 136 (10), 1416–1424. https://doi.org/10.1017/S0950268807000039.

Dai, X., Shang, G., Lu, S., Yang, J., Xu, J., 2018. A new subtype of eastern tick-borne encephalitis virus discovered in Qinghai-Tibet Plateau, China. Emerg Microbes Infect 7 (1), 74. https://doi.org/10.1038/s41426-018-0081-6.

Dekker, M., Laverman, G.D., de Vries, A., Reimerink, J., Geeraedts, F., 2019. Emergence of tick-borne encephalitis (TBE) in the Netherlands. Ticks Tick Borne Dis 10 (1), 176–179. https://doi.org/10.1016/j.ttbdis.2018.10.008.

Demina, T.V., Dzhioev, Y.P., Verkhozina, M.M., Kozlova, I.V., Tkachev, S.E., Plyusnin, A., Doroshchenko, E.K., Lisak, O.V., Zlobin, V.I., 2010. Genotyping and characterization of the geographical distribution of tick-borne encephalitis virus variants with a set of molecular probes. J Med Virol 82 (6), 965–976. https://doi.org/10.1002/jmv.21765.

Dobler, G., Gniel, D., Petermann, R., Pfeffer, M., 2012. Epidemiology and distribution of tick-borne encephalitis. Wien Med Wochenschr 162 (11-12), 230–238. https://doi.org/10.1007/s10354-012-0100-5.

Ecker, M., Allison, S.L., Meixner, T., Heinz, F.X., 1999. Sequence analysis and genetic classification of tick-borne encephalitis viruses from Europe and Asia. J Gen Virol 80 (Pt 1), 179–185. https://doi.org/10.1099/0022-1317-80-1-179.

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32 (5), 1792–1797. https://doi.org/10.1093/nar/gkh340.

Grubaugh, N.D., Gangavarapu, K., Quick, J., Matteson, N.L., De Jesus, J.G., Main, B.J., Tan, A.L., Paul, L.M., Brackney, D.E., Grewal, S., Gurfield, N., Van Rompay, K.K.A., Isern, S., Michael, S.F., Coffey, L.L., Loman, N.J., Andersen, K.G., 2019. An amplicon-based sequencing framework for accurately measuring intrahost virus diversity using PrimalSeq and iVar. Genome Biol 20 (1), 8. https://doi.org/10.1186/s13059-018-1618-7.

Haglund, M., Vene, S., Forsgren, M., Gunther, G., Johansson, B., Niedrig, M., Plyusnin, A., Lindquist, L., Lundkvist, A., 2003. Characterisation of human tick-borne encephalitis virus from Sweden. J Med Virol 71 (4), 610–621. https://doi.org/10.1002/jmv.10497.

Heinz, F.X., Kunz, C., 1981. Homogeneity of the structural glycoprotein from European isolates of tick-borne encephalitis virus: comparison with other flaviviruses. J Gen Virol 57 (Pt 2), 263–274. https://doi.org/10.1099/0022-1317-57-2-263.

Heinz, F.X., Mandl, C.W., 1993. The molecular biology of tick-borne encephalitis virus.

Heinze, D.M., Gould, E.A., Forrester, N.L., 2012. Revisiting the clinal concept of evolution and dispersal for the tick-borne flaviviruses by using phylogenetic and biogeographic analyses. J Virol 86 (16), 8663–8671. https://doi.org/10.1128/jvi.01013-12.

Holding, M., Dowall, S.D., Medlock, J.M., Carter, D.P., McGinley, L., Curran-French, M., Pullan, S.T., Chamberlain, J., Hansford, K.M., Baylis, M., Vipond, R., Hewson, R., 2019. Detection of new endemic focus of tick-borne encephalitis virus (TBEV), Hampshire/Dorset border, England, September 2019. Euro Surveill 24 (47). https://doi.org/10.2807/1560-7917.Es.2019.24.47.1900658.

Holzmann, H., Aberle, S.W., Stiasny, K., Werner, P., Mischak, A., Zainer, B., Netzer, M., Koppi, S., Bechter, E., Heinz, F.X., 2009. Tick-borne encephalitis from eating goat cheese in a mountain region of Austria. Emerg Infect Dis 15 (10), 1671–1673. https://doi.org/10.3201/eid1510.090743.

Hudopisk, N., Korva, M., Janet, E., Simetinger, M., Grgic-Vitek, M., Gubensek, J., Natek, V., Kraigher, A., Strle, F., Avsic-Zupanc, T., 2013. Tick-borne encephalitis associated with consumption of raw goat milk, Slovenia, 2012. Emerg Infect Dis 19 (5), 806–808. https://doi.org/10.3201/eid1905.121442.

Kaufusi, P.H., Kelley, J.F., Yanagihara, R., Nerurkar, V.R., 2014. Induction of endoplasmic reticulum-derived replication-competent membrane structures by West Nile virus non-structural protein 4B. PLoS One 9 (1), e84040. https://doi.org/10.1371/journal.pone.0084040.

Kerlik, J., Avdicova, M., Stefkovicova, M., Tarkovska, V., Pantikova Valachova, M., Molcanyi, T., Mezencev, R., 2018. Slovakia reports highest occurrence of alimentary tick-borne encephalitis in Europe: Analysis of tick-borne encephalitis outbreaks in Slovakia during 2007-2016. Travel Med Infect Dis. https://doi.org/10.1016/j.tmaid.2018.07.001.

Klaus, C., Gethmann, J., Hoffmann, B., Ziegler, U., Heller, M., Beer, M., 2016. Tick infestation in birds and prevalence of pathogens in ticks collected from different places in Germany. Parasitol Res 115 (7), 2729–2740. https://doi.org/10.1007/s00436-016-5022-5.

Ko, S., Kang, J.G., Kim, S.Y., Kim, H.C., Klein, T.A., Chong, S.T., Sames, W.J., Yun, S.M., Ju, Y.R., Chae, J.S., 2010. Prevalence of tick-borne encephalitis virus in ticks from southern Korea. J Vet Sci 11 (3), 197–203. https://doi.org/10.4142/jvs.2010.11.3.197.

Kovalev, S.Y., Mukhacheva, T.A., 2017. Reconsidering the classification of tick-borne encephalitis virus within the Siberian subtype gives new insights into its evolutionary history. Infect Genet Evol 55, 159–165. https://doi.org/10.1016/j.meegid.2017.09.014.

Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. Nature methods 9 (4), 357–359. https://doi.org/10.1038/nmeth.1923.

Li, H., 2011. A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. Bioinformatics 27 (21), 2987–2993. https://doi.org/10.1093/bioinformatics/btr509.

Lindquist, L., Vapalahti, O., 2008. Tick-borne encephalitis. Lancet 371 (9627), 1861–1871. https://doi.org/10.1016/s0140-6736(08)60800-4.

Mandl, C.W., Kunz, C., Heinz, F.X., 1991. Presence of poly(A) in a flavivirus: significant differences between the 3' noncoding regions of the genomic RNAs of tick-borne encephalitis virus strains. J Virol 65 (8), 4070–4077.

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 17 (1), 10. https://doi.org/10.14806/ej.17.1.200.

Mlera, L., Bloom, M.E., 2018. The Role of Mammalian Reservoir Hosts in Tick-Borne Flavivirus Biology. Front Cell Infect Microbiol 8, 298. https://doi.org/10.3389/fcimb.2018.00298.

Nguyen, L.-T., Schmidt, H.A., von Haeseler, A., Minh, B.Q., 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum likelihood phylogenies. Mol. Biol. Evol. 32, 268–274. https://doi.org/10.1093/molbev/msu300.

Nuttall, P.A., Labuda, M., 2003. Dynamics of infection in tick vectors and at the tick-host interface. Adv Virus Res 60, 233–272. Retrieved from. https://www.ncbi.nlm.nih.gov/pubmed/14689696.

Paulsen, K.M., Stuen, S., das Neves, C.G., Suhel, F., Gurung, D., Soleng, A., Stiasny, K., Vikse, R., Andreassen, A.K., Granquist, E.G., 2019. Tick-borne encephalitis virus in cows and unpasteurized cow milk from Norway. Zoonoses Public Health 66 (2), 216–222. https://doi.org/10.1111/zph.12554.

Plaszczyca, A., Scaturro, P., Neufeldt, C.J., Cortese, M., Cerikan, B., Ferla, S., Brancale, A., Pichlmair, A., Bartenschlager, R., 2019. A novel interaction between dengue virus nonstructural protein 1 and the NS4A-2K-4B precursor is required for viral RNA replication but not for formation of the membranous replication organelle. PLoS Pathog 15 (5), e1007736. https://doi.org/10.1371/journal.ppat.1007736.

Potapov, V., Ong, J.L., 2017. Examining Sources of Error in PCR by Single-Molecule Sequencing. PLoS One 12 (1). https://doi.org/10.1371/journal.pone.0169774 e0169774.

Quick, J., Grubaugh, N.D., Pullan, S.T., Claro, I.M., Smith, A.D., Gangavarapu, K., Oliveira, G., Robles-Sikisaka, R., Rogers, T.F., Beutler, N.A., Burton, D.R., Lewis-Ximenez, L.L., de Jesus, J.G., Giovanetti, M., Hill, S.C., Black, A., Bedford, T., Carroll, M.W., Nunes, M., Alcantara Jr., L.C., Sabino, E.C., Baylis, S.A., Faria, N.R., Loose, M., Simpson, J.T., Pybus, O.G., Andersen, K.G., Loman, N.J., 2017. Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. Nature Protocols 12, 1261. https://doi.org/10.1038/nprot.2017.066.

Romanova, L., Gmyl, A.P., Dzhivanian, T.I., Bakhmutov, D.V., Lukashev, A.N., Gmyl, L. V., Rumyantsev, A.A., Burenkova, L.A., Lashkevich, V.A., Karganova, G.G., 2007. Microevolution of tick-borne encephalitis virus in course of host alternation. Virology 362 (1), 75–84. https://doi.org/10.1016/j.virol.2006.12.013.

Ruzek, D., Avsic Zupanc, T., Borde, J., Chrdle, A., Eyer, L., Karganova, G., Kholodilov, I., Knap, N., Kozlovskaya, L., Matveev, A., Miller, A.D., Osolodkin, D.I., Overby, A.K., Tikunova, N., Tkachev, S., Zajkowska, J., 2019. Tick-borne encephalitis in Europe and Russia: Review of pathogenesis, clinical features, therapy, and vaccines. Antiviral Res 164, 23–51. https://doi.org/10.1016/j.antiviral.2019.01.014.

Saksida, A., Duh, D., Lotric-Furlan, S., Strle, F., Petrovec, M., Avsic-Zupanc, T., 2005. The importance of tick-borne encephalitis virus RNA detection for early differential diagnosis of tick-borne encephalitis. J Clin Virol 33 (4), 331–335. https://doi.org/10.1016/j.jcv.2004.07.014.

Slavik, I., Mrena, E., Mayer, V., 1970. Studies on Tick-borne encephalitis virus. II. Virus morphology and some data on virus structure. Acta Virol 14 (1), 8–16.

Smura, T., Tonteri, E., Jaaskelainen, A., von Troil, G., Kuivanen, S., Huitu, O., Kareinen, L., Uusitalo, J., Uusitalo, R., Hannila-Handelberg, T., Voutilainen, L., Nikkari, S., Sironen, T., Sane, J., Castren, J., Vapalahti, O., 2019. Recent establishment of tick-borne encephalitis foci with distinct viral lineages in the Helsinki area, Finland. Emerg Microbes Infect 8 (1), 675–683. https://doi.org/10.1080/22221751.2019.1612279.

Stiasny, K., Holzmann, H., Heinz, F.X., 2009. Characteristics of antibody responses in tick-borne encephalitis vaccination breakthroughs. Vaccine 27 (50), 7021–7026. https://doi.org/10.1016/j.vaccine.2009.09.069.

Suss, J., 2011. Tick-borne encephalitis 2010: epidemiology, risk areas, and virus strains in Europe and Asia-an overview. Ticks Tick Borne Dis 2 (1), 2–15. https://doi.org/10.1016/j.ttbdis.2010.10.007.

Velay, A., Solis, M., Kack-Kack, W., Gantner, P., Maquart, M., Martinot, M., Augereau, O., De Briel, D., Kieffer, P., Lohmann, C., Poveda, J.D., Cart-Tanneur, E., Argemi, X., Leparc-Goffart, I., de Martino, S., Jaulhac, B., Raguet, S., Wendling, M.J., Hansmann, Y., Fafi-Kremer, S., 2018. A new hot spot for tick-borne encephalitis (TBE): A marked increase of TBE cases in France in 2016. Ticks Tick Borne Dis 9 (1), 120–125. https://doi.org/10.1016/j.ttbdis.2017.09.015.

Waldenström, J., Lundkvist, A., Falk, K.I., Garpmo, U., Bergström, S., Lindegren, G., Sjöstedt, A., Mejlon, H., Fransson, T., Haemig, P.D., Olsen, B., 2007. Migrating birds and tickborne encephalitis virus. Emerg Infect Dis 13 (8), 1215–1218. https://doi.org/10.3201/eid1308.061416.

Yoshii, K., Song, J.Y., Park, S.B., Yang, J., Schmitt, H.J., 2017. Tick-borne encephalitis in Japan, Republic of Korea and China. Emerg Microbes Infect 6 (9), e82. https://doi.org/10.1038/emi.2017.69.

Yun, S.M., Kim, S.Y., Ju, Y.R., Han, M.G., Jeong, Y.E., Ryou, J., 2011. First complete genomic characterization of two tick-borne encephalitis virus isolates obtained from wild rodents in South Korea. Virus Genes 42 (3), 307–316. https://doi.org/10.1007/s11262-011-0575-y.

Paper IV, Supplementary table 1

Table 1: Ambiguous nucleotides (<2%) for the virus strains Hochosterwitz and 1997/783 compared with their respective consensus genome

Position Hochosterwitz*	Region**	Coverage	Ambiguous nucleotides (variant frequency)	Amino acid change
21	NCR	20217	T→C (25.1%)	NCR
23	NCR	20316	G→A (25.1%)	NCR
186	Capsid	20367	T→C (24.7%)	Same
250	Capsid	18414	C→T (25.6%)	**Leu→Ser y (transition)**
276	Capsid	16853	T→C (22.7%)	Same
300	Capsid	21299	A→G (18.4%)	Same
334	Capsid	23295	G→A (17.9%)	Val→Ile r (transition)
339	Capsid	23216	C→T (4.8%)	Same
354	Capsid	23416	G→A (2.4%)	Same
399	Capsid	18634	T→C (21.8%)	**Tyr→His y (transition)**
522	Pr. membrane	17450	C→T (25.7%)	Same
528	Pr. membrane	17159	T→C (25.7%)	Same
562	Pr. membrane	16513	G→T (25.3%)	**Ala→Ser k (transversion)**
759	Membrane	13305	T→C (26.5%)	Same
783	Membrane	13527	A→G (26.6%)	Same
831	Membrane	13398	C→T (27.4%)	Same
855	Membrane	13343	A→G (26.9%)	Same
858	Membrane	13344	C→T (26.9%)	Same
897	Membrane	13302	T→ C (25.6%)	Same
898	Membrane	13275	T→C (25.6%)	Same
1008	Envelope	13456	C→T (27.1%)	Same
1044	Envelope	13174	G→A (27.5%)	Same
1053	Envelope	13350	T→C (27.2%)	Same
1077	Envelope	13266	T→C (27.3%)	Same
1152	Envelope	13526	G→A (27.6%)	Same
1167	Envelope	13692	A→G (27.2%)	Same
1179	Envelope	13716	C→T (27.5%)	Same
1329	Envelope	15049	A→G (25.6%)	Same
1386	Envelope	15318	C→T (26.8%)	Same
1407	Envelope	15785	C→T (27.0%)	Same
1419	Envelope	15700	A→T (26.3%)	Same
1435	Envelope	15651	T→C (26.8%)	Same
1440	Envelope	15536	C→T (26.9%)	Same
1464	Envelope	14891	T→C (27.1%)	Same
1503	Envelope	14631	G→A (28.6%)	Same
1506	Envelope	14659	C→T (28.6%)	Same
1616	Envelope	15822	A→C (25.9%)	**Asn→Thr m (transversion)**
1746	Envelope	55618	A→G (25.4%)	Same
1767	Envelope	58355	G→A (25.9%)	Same
1797	Envelope	61695	A→G (25.8%)	Same
1821	Envelope	61892	T→C (26.8%)	Same
1875	Envelope	57905	T→C (27.4%)	Same
1887	Envelope	56154	C→T (27.6%)	Same

1923	Envelope	50913	A→G (46.1%)	Same (reverse primer 1)
3290	NS1	33633	G→T (11.4%)	**Gly→Val k (transversion)**
6569	NS4A	31961	T→C (2.4%)	**Val→Ala y (transition)**
6633	NS4A	93546	G→A (28.8%)	Same
6757	NS4A	134707	C→T (3.7%)	Same
9711	NS5	107274	T→A (2.6%)	**Phe→Leu w (transversion)**
10591	NCR	12771	G→A (3.3%)	NCR
10650	NCR	15066	T→C (3.5%)	NCR
10866	NCR	46679	C→T (3.0%)	NCR

Position 1993/783*	**Region****	**Coverage**	**Ambiguous nucleotides (variant frequency)**	**Amino acid change**
3042	NS1	24742	T→C (25.6%)	Same
3148	NS1	27381	G→A (5.0%)	**Ala→Thr r (transition)**
3290	NS1	37129	G→T (6.7%)	**Gly→Val k (transversion)**
6221	NS3	67624	C→T (26.8%)	**Thr→Met y (transition)**
8337	NS5	67537	T→C (33.7%)	Same
8927	NS5	61799	G→A (2.9%)	**Arg→Lys r (transition)**
10114	NS5	118538	G→T (2.6%)	**Asp→Tyr k (transversion)**
10865	NCR	5114	A→G (10.0%)	NCR

The positions correspond to the respective strains consensus sequence. GenBank Accession number: MT311861.1 and MT311860.1

**NCR: non coding region, NS: non-structural proteins

*** Leu= Leucine, Ser=Serine, Val=Valine, Ile=Isoleucine, Tyr=Tyrosine, His=Histidine, Ala=Alanine, Asn=Asparagine Thr= Threonine, Gly=Glycine, Met=Methionine, Arg= Arginine, Lys= Lysine, Phe= Phenylalanine, Asp= Aspartic Acid. Amino acids with different function are in bold

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Doctoral dissertations at the University of South-Eastern Norway no. 190

ISBN 978-82-7206-840-9 (print)
ISBN 978-82-7206-841-6 (online)

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