# Tick-borne encephalitis virus in Alpine regions

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For those who had to leave too soon.

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# ABBREVIATIONS

| | |
|---|---|
| TBE | Tick-borne encephalitis |
| NS | Non-structural protein |
| prM | Pre Membrane glycoprotein |
| M | Membrane glycoprotein |
| C | Capsid protein |
| E | Envelope protein |
| I. | Ixodes |

# I. Introduction

Infectious diseases are ubiquitous in nature and have always affected humans. Particularly zoonotic diseases transmitted from wildlife or livestock can have dramatic impacts on human societies and health (e.g. avian flu, yellow fever), with the most recent example being the currently ongoing pandemic of Sars-CoV-2, which began in 2019 and has since spread around the globe.
A range of zoonotic and vector borne diseases, including Malaria, West-Nile-Virus and Zika-Virus are increasing their prevalence and geographic range, which is attributable to anthropogenic changes in the environment. Increasing habitat fragmentation, for instance due to deforestation, facilitates closer contact between livestock, wildlife and humans. Climate change leads to changes in the environment, facilitating range-expansions for common vector species of vector borne diseases e.g. Asian tiger mosquitoes (*Aedes albopictus*), potential vectors for Zika-Virus or Dengue-Virus found in Germany. While the general patterns leading to an increased risk of transmission of zoonotic diseases based on man-made environmental changes are clear, the precise mechanisms driving the expansion of the geographic range of many infectious diseases, particularly the adaptations of both vector and pathogen to new habitats or environmental condition, are not yet well understood for many systems.
One such system is tick-borne-encephalitis, a viral infection transmitted by ticks, which's endemic range has been reported to be increasing.

Tick-borne encephalitis is an infectious, zoonotic disease caused by the tick-borne encephalitis virus (TBEV) which can infect humans and animals. Usually, it is transmitted via tick bites of the Ixodidae family (*Ixodes spp.)*, but some outbreaks after ingestion of unpasteurised milk from viraemic livestock animals have also been reported (Kriz et al 2009, Hudopisk et al 2013, Holzmann et al 2009). Usually, clinical signs of infections are meningitis-like headaches with or without neuronal deficits, encephalomyelitis or meningitis, with a low mortality resulting from infections; of 445 cases reported with both clinical signs and laboratory confirmation of TBE infection in Germany in 2019, only two patients died from TBE (RKI 2019).

This virus is not only present in Germany. Linked to the distribution of its most common vector Ixodidae ticks, it is found in large parts of Eurasia. In recent years a change in geographical and altitudinal distribution of reported TBE cases has been observed in several countries all

over Europe (Heinz et al 2015, Zeman and Beneš 2004). The TBE virus infections could be linked to regions formerly not noted as endemic areas. The shift of TBE cases in Austria has been well described by Heinz et al 2015 where the number of reported outbreaks started to show a change in distribution from lower altitudinal regions like Styria and Carinthia into regions higher above sea level like Tyrol and Vorarlberg. Reporting and exploring this distributional change is especially important for vaccination advice for locals, tourists and hunters. The cause of this shift is of date not yet explored.

There are various factors which could contribute to these new occurrences. The following hypotheses are considered an explanation: First, the virus could have either adapted to the environment found in higher altitudes in the Alps or a single progenitor strain could have spread into further regions. Second, the environment could have changed through introduction of new plant species or reservoir animals, as well as the overall climate in the Alpine region. Third, the virus could have spread through a different tick species or an adaptation of the current vector to the climate of higher altitude Alpine regions. Lastly, changes in human activity in Alpine regions could warrant the observed changes.
This work focuses on the TBEV and its genetics. Earlier works concentrated mostly on the number of infections, the number of ticks and infected ticks. While previous studies linked the incidence of infections with the number of infected ticks, as of now the role of different virus strains has not been investigated further. Consequently, the aim of my study was to determine whether genetic differences between viral strains can explain the observed change in altitudinal distribution. We compared virus strains from regions of various altitudes to assess the possible connection between the establishment of new foci in mountainous regions and the respective newly occurring virus strains.

# II. Tick-borne Encephalitis (TBE)

## 1. Tick-borne encephalitis virus (TBEV)

### 1.1. TBEV - a *Flavivirus*

The genus *Flavivirus* is a member of the family of *Flaviviridae*. Flaviviruses have a single-stranded RNA genome of positive polarity (Westaway 1969/2011, Westaway et al 1985/2013). Most flaviviruses are known vector-borne viruses that are transmitted to their intended host via mosquitoes or tick bites (Pierson and Diamond 2013). The family contains over 70 virus species, including a number of important animal and human pathogens, like dengue virus, West Nile virus, yellow fever or Zika virus, all transmitted via mosquitoes (Růžek et al 2018, WHO 2017, WHO 2018, WHO 2019, WHO 2020). TBEV and Louping ill virus, which are transmitted via tick bites (Gould and Solomon 2008), also belong to the family of Flaviviridae. The prototype virus of the family Flaviviridae is the yellow fever virus which also gave the family their name (Westaway et al 1985/2013), flavus being the Latin word for yellow. Their zoonotic potential is notable since they are able to infect a wide range of vertebral hosts (Westaway et al 1985/2013). In recent years, numerous flaviviruses have expanded their endemic areas, posing an important threat to animal and public health (Hollidge et al 2010, Wilson 2013).

### 1.2. Virus morphology and genetics

Tick-borne encephalitis virus is a member of the genus *Flavivirus.* Flaviviruses have a lipid capsule (Westaway 1969/2011, Westaway et al 1985/2013). Their structural proteins are located at the 5’ terminus and the RNA-synthesis is performed perinuclear in infected cells (Westaway et al 1985/2013). Under an electron microscope, the TBE virus appears as a spherical particle, with diameters ranging between 45 nm and 50 nm (Westaway 1969/2011). The capsule is an icosahedral structure and the virus itself possesses 3 structural proteins: core protein C, which contains the virus genome, the membrane proteins (prM or M) and the glycoprotein E (Westaway et al 1985/2013). The most important proteins for virus attachment in mature virus particles are the M protein and the glycoprotein E, which form the virus capsule together with lipids and polysaccharides (Heinz and Mandl 1993, Heinz and Allison 2003).

TBE virus genomes of different strains show a varying length but a single ORF (open reading frame). While the 5’ end is highly conserved for the length of 132 nucleotides,the 3’ end has

conserved and variable parts and can show length polymorphism. The ORF has a constant length and is translated into a single polyprotein, which is divided into its different components by viral and cellular proteases. The codons for the structural proteins, protein C, prM/M and E, are located in the first quarter of the ORF, while the remaining three quarters code for non-structural proteins (Chambers et al 1990, Westaway et al 1985/2013).

Different virus subtypes of TBE virus have emerged over the centuries, currently differentiated in three subtypes: the European, the Far-Eastern and the Siberian subtype (Heinz and Kunz 1982). There have been claims of two additional subtypes, the Baikal subtype and the Himalayan subtype (Tkachev et al 2017, Dai et al 2018). The different subtypes differ in their respective virulence and the clinical symptoms they cause. A further investigation into the nucleotide and amino acid sequences of the protein E of the different subtypes showed the three established subtypes have very low differentiation of 3,6 - 5,6 % between themselves (Holzmann et al 1997, Ecker et al 1999). Each subtype in turn includes a range of different virus strains which show different qualities in neurovirulence as well as nucleotide and amino acid sequence.

## 1.3. Viral proteins

Point mutations in the viral genome can lead to changes in the viral proteins. Subsequently, this can lead to different morbidity and mortality. Therefore, it is important to understand the role of each protein, structural and non-structural.
TBE possesses seven non-structural proteins, namely NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Heinz et Mandl 1993). NS1 is peripherally associated with membranes (Lindenbach and Rice 2003, Muller and Young 2013). It is essential for virus replication and also thought to interact with translation and virion production (Cervantes-Salázar et al 2015). It is also known to inhibit the complement system of the host immune response by Toll-like receptor activation (Avirutnan et al 2011, Rastogi et al 2016). NS2A is small and its role is yet unclear (Lindenbach and Rice 2003) whereas, NS2B is important for the activity of the protease of NS3 (Chambers at al 1993), which is an essential enzyme for virus replication and polyprotein processing (Lindenbach and Rice 2003). The small hydrophobic proteins NS4A and NS4B are believed to play a role in virus replication (Uchil and Satchidanandam 2003). And the rather large NS5 protein serves as a viral RNA-dependent RNA polymerase (Muñoz-Jordán et al 2003).

As stated above besides protein C two more structural proteins exist. The prM protein shows a chaperone-like capacity in the folding of protein E (Goto et al 2005). The cleavage of prM

into M is performed in the Golgi apparatus of infected cells and the pr component is secreted afterwards (Elshuber et al 2003, Stadler et al 1997, Lindenbach and Rice 2003). Protein E contains the major viral antigens and mitigates both receptor-specific binding to as of yet unknown receptors to cells and the entry into host cells (Gritsun et al 1995).

Several experiments so far show that the different virulence of TBE virus strains depends on a range of factors, with structural changes in protein E playing a major role in virus attachment. Different regions of the protein E, as well as the capsular protein and the genome have been linked to variation in virulence. For protein E, domain III is a highly notable factor for the virulence, as well as hidden regions between domain I and II, the tip of domain II and its junctures to other dimers (Holzmann et al 1997). Moreover, non-structural proteins play a role at immune evasion and virus assembly. The amino acid position 310 is an important factor in the genome for higher or lower virulence. For example, a replacement of the amino acid sequence at position 310 for lysine results in a lower virulence (Satz 2006).

## 1.4. Virus replication

The virus enters cells via receptor-mediated endocytosis, through binding of protein E to an to date still unidentified receptor. With involvement of multiple rather than only one specific receptor facilitating virus entry into the cell being a likely scenario (Kopecký et al 1999, Kroschewski et al 2003). Notably, the role of glycosaminoglycans, most notably heparan sulfate in facilitating receptor binding has been discussed. Glycosaminoglycans are ubiquitous present on cell surfaces and may be used to facilitate a higher virus concentration on the cell surface (Bernfield et al 1999). After endocytosis, the virus enters the cell via a vesicle fusing with the cellular endosome: The acidic milieu within the endosomal vesicle triggers a conformational change in protein E from its dimeric form to a trimeric form, which initiates the fusion of the viral envelope with the vesicular membrane (Alen and Schols 2012), releasing the viral genome into the cytoplasm, where the positively charged RNA of the TBE virus can directly act as an translational template. Afterwards, a preliminary polyprotein will be translated from the viral RNA, which is then cleaved into the respective viral proteins (Mandl 2005). Lastly, the virus assembly takes place inside the endoplasmatic reticulum (Muller and Young 2013), where a preliminary non-infective virion is formed with the structural proteins prM and E in a heterodimeric association. The maturation of the virus through the synthesis of prM to M happens afterwards in the Golgi apparatus, which is likely facilitated by a cellular furin-like protease, though the exact process is still unknown (Mandl 2005). Normally, infected host cells die by apoptosis or necrosis, but persistent infections for the tick-borne flavivirus Langat virus in cell culture were possible (Mlera et al 2015).

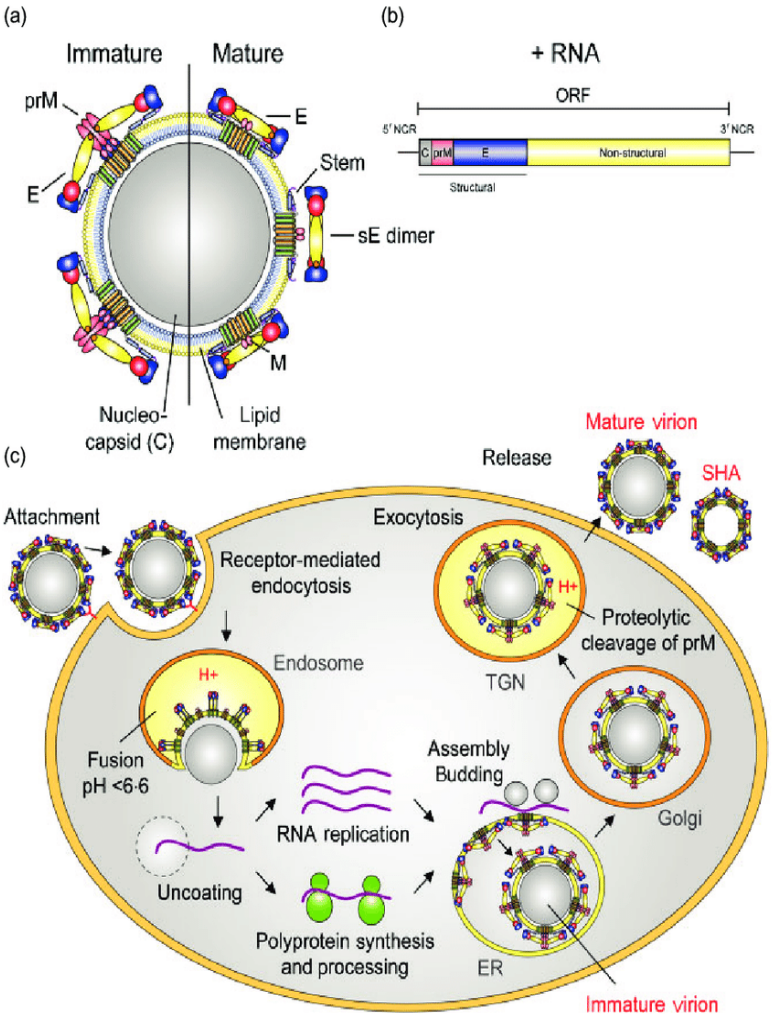


Fig. 1: *Schematic representations of flavivirus particles (a), their genome organization (b) and the viral life cycle (c). (Stiasny and Heinz 2006)*

## 1.5. Tenacity

Due to the lipid capsule, the TBE virus can be inactivated with organic solutions. While it is not heat-stable (Satz 2006, Gresikova-Kohutova 1959), deep freezing apparently does not harm the virus (Gritsun et al 1995). The TBE virus is capable of surviving in aerosols, liquids and powders, where it can stay stable and infectious for up to six hours at room-temperature when the humidity is between 23 and 80%. The TBE virus is stable nearly indefinitely at room temperature after dry freezing.

The virus can be inactivated through UV light and ɣ-radiation, as well as 3-4% of formaldehyde, 2% of glutaraldehyde, 2-3% of hydrogen peroxide, chlorine (500-5000 ppm), alcohol, 1% of iodine or phenol iodophor (Satz 2006, Gritsun et al 2003).

# 2. Ticks as most important TBE virus vectors

## 2.1. Tick anatomy and biology

Ticks are important vectors for a great number of zoonotic diseases, like Lyme disease, Rickettsiosis, Crim-Congo Haemorrhagic fever and TBE, with the most important TBE virus vector in Western Europe being the castor bean tick *Ixodes ricinus*.

Ticks belong to the class of *Arachnidae*, subclass *Acarida* and order of *Ixodida*. They are divided into three families, hard ticks, *Ixodidae*, soft ticks, *Argasidae* and the monotypic *Nutalliellidae*. For this work only hard ticks were of interest.

The ticks found in Western and Central Europe are as big as the back of a roller point pen in its unfed adult stage and the size of a poppy seed in its nymphal stage. These ticks are characterised by a distinctive anal groove posterior of the anus.

Ticks have three leg pairs in their larval stage and four in the nymphalid and adult stages. Their rostrum, comparable with a mammal's mouth, consists of three parts: The ventral hypostome with teeth, and a dorsal pair of chelicerae with cutting tools on their tips for the perforation of skin. These three parts form a canal functioning as a mouth. The saliva glands are most important for the water and electrolyte balance of the ticks. The grape-like structure enlarges greatly while feeding. The tick gut is composed of different diverticula, which are capable of containing large amounts of blood. The enormous enlargement of ticks is only possible because the tick's alloscutum, its skin, is folded like an accordion, allowing for massive expansion upon a blood meal (Mehlhorn 1996).

*Ixodes ricinus* has its sensory organs, called sensilla, all over its body. The most sensilla are located at the tarsus of the first pair of legs, where they form the Haller Organ, which is a key organ for environmental perception, including vibrations, odours, pheromones, temperature, and humidity. They are made aware of a potential host by the vibrations caused when hosts touch bushes and grass and – most importantly – the amount of $CO_2$ in their surroundings (Waladde and Rice 1982, Sonenshine et al 2002, Guetard 2001, Mehlhorn 2001).

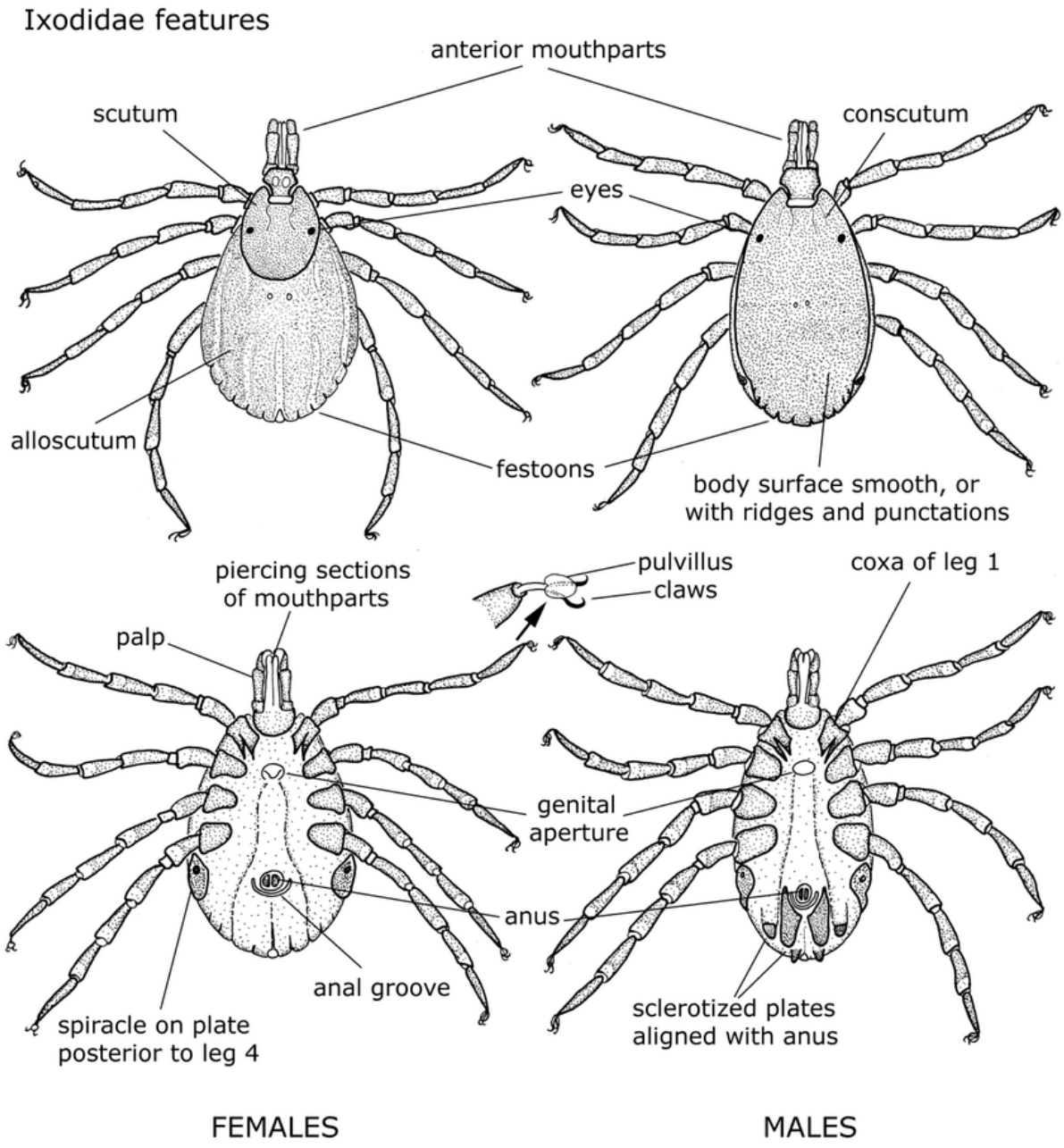


Fig. 2: *Morphological features of hard ticks (family Ixodidae). Example is an adult female and an adult male of the genus Hyalomma. Top row is dorsal view, bottom row is ventral view. (Barker and Walker 2014)*

After hatching, the ticks need to feed on blood for moulting into the next stage. In *Ixodes* ticks, the female needs to feed on blood for the production of the eggs, while the sperms of their male counterpart have already evolved in the nymphalid stage, so males do not necessarily need to feed on blood in their adult stage (Aeschlimann 1991). The male tick dies after copulation and the female after laying the eggs. Since they coat every egg with wax produced from the porose areas on their dorsal scutum, egg laying can be a quite time-consuming process and can take up to 20 days per female (Aeschlimann 1996, Matuschka 1996).

Ticks live on meadows and in forest underbrush, where they stay close to the ground. A high humidity of approximately 80% is necessary for their survival, yet too high humidity can impair their survival (Sonenshine et al 2002). When questing they climb the surrounding vegetation. Larvae are found up to 30 cm, nymphs just short of 1 m and adults up to 1,5 m above the ground (Liebisch and Liebisch 2003). *Ixodes* ticks, contrary to other species, which are actively hunting their host, are waylaying ticks, waiting for a potential host to pass by. When they sense the vibrations, indicating a potential host, they drop down blindly, not knowing if they will drop onto the passing creature. Due to the relatively high humidity, wooded areas, namely forest outskirts, bordering a meadow are the preferred habitat for ticks in Central Europe (Radda 1969).

## 2.2. TBE virus transmission and adaptation to ticks

The TBE virus enters the tick gut either within infected host cells or as viral particles in blood via a co-feeding mechanism during a blood meal. Not every virus is capable of passing through the gut cell barrier (Nutall et al 1994). There are different possibilities how a tick can be infected with the TBE virus. One possibility is the acquisition through a host with a high viremia of the TBE virus. The hosts with the highest viremia are mostly small rodents, namely *Apodemus flavicollis* and *Myodes glareolus* (Radda 1969). Another, important mechanism, is the co-feeding of ticks in near proximity (about 1 cm apart) on the same host. The blood lagoons merge and secreted pathogens can travel into other ticks. This is possible, even if the tick host is immune to the TBE virus (Labuda et al 1997, Korenberg 1994). TBE resides primarily in the salivary glands of ticks. Unlike other pathogens transmitted by ticks, like *Borellia spp.*, which reside in the tick's gut and therefore, require prolonged attachment to the host, TBE can thus be transmitted through the coxal glands very quickly (Mehlhorn 1996).

The tick metamorphosis is a critical point and the virus seems to retreat into tissues that are stable during moulting - otherwise, the virus titre has to be able to re-establish itself. There is evidence that once infected ticks most likely stay infected until the end of their life (Nutall et al 1994). Furthermore, the virus seems to show adaptations to the tick it has already established as host. This was demonstrated experimentally by a study by Růžek et al (2008), when they tested the amount of virus produced in host and non-host tick cell lines.

# 3. Tick-borne encephalitis

## 3.1. TBE in humans

There is strong evidence of asymptomatic infections where people are seropositive but show no signs of clinical disease. The most evidently targeted organ of a TBE virus infection is the central nervous system. Parts of the diencephalon, the cerebrum, the brainstem and the cervical cores appear to be the primarily infected structures. After a subcutaneous multiplication, the TBE virus spreads sub-clinically via the lymphatic system and later on haematogenous before causing symptoms when infecting specific brain structures (Satz, 2006).

Signs of TBE infections are an elevated cell count and a higher level of glucose in liquor samples and leucocytosis with left deviation in blood, usually accompanied by blood sedimentation. The TBE infection can be divided into three periods: the prodromal period, the in-between period and the main period, which are described in more detail next.

First is the prodromal period which encompasses the first stage of the TBE infection and consequently, the initial viremia. According to the observations of Kaiser (1999) in 485 patients with a biphasic course, the prodromal period showed a duration of two to eight days, with a median of four days. The clinical signs of a patient in the prodromal period are not specific for a TBE virus infection, including fatigue, myalgia and flu-like symptoms. Serology for the TBE virus is negative and the liquor findings are physiological (Grinschgl 1955, Ackermann et al 1979) at this stage. Extra neural organ manifestations occur primarily in this period. Compared to the central nervous infection, the prodromal phase plays only a minor role in clinical cases. There are some hypotheses about an abortive TBE virus infection only encompassing a prodromal period, possibly because the virus is not able to overcome the blood-brain barrier. Its existence is highly debated and different authors claim a percentage of 4% to 2/3$^{rd}$ of the cases to be abortive (Grinschgl 1955, Conrads and Plassmann 1982, Kunz 1992, Ackermann et al 1986, Lotric-Furlan et al 2002).

This is followed by the In-between period which links the prodromal period and the main phase, in which hardly any symptoms are displayed, leading to a biphasic course. This phase usually lasts between seven to twelve days, but a period of up to three weeks has been reported (Satz 2006). Sometimes a general unwell feeling, as well as myalgias and mild headaches persist in this phase (Grinschgl 1955, Wahlberg et al 1989). In case of a monophasic course, clinical ailments remain present throughout the in-between period.

Lastly follows the main period which is usually characterized by central-nervous symptoms. It can be classified according to the primarily affected CNS regions, with three classifications: the meningitic paralytic or non-paralytic forms, the spinal, bulbospinal, ascending, bulbar, radiculitic and trans-myelitic paralytic forms and the encephalitic form, which were defined by Grinschgl (1955) based on 304 clinical patients. Most publications differentiate meningitis, meningoencephalitis and meningo encephalo radiculitis (Ackermann et al 1970, Ackermann et al 1979, Duniewicz 1979, Köck et al 1992, Krech et al 1969), sometimes with the addition of the encephalomyelitis. Some authors classify TBE as mild, moderate and severe cases (Günther et al 1997, Mickiene et al 2002, Bohr et al 1985, Kaiser et al 1997, Kaiser 2000), irrespective of the affected parts of the CNS. Since there are different classifications, the recorded manifestation percentages vary, but in Western and Central Europe, meningitis seems to be the most abundant clinical course in humans. Since the main phase can take different courses of illness, the convalescence varies greatly (Satz 2006). The more severe the clinically manifested TBE is, the longer the recovery lasts and the more likely sequelae occur (Mickiene 2002, Kaiser 1999, Kaiser et al 1997).

Furthermore, the clinical outcome is a matter of age. Mostly, a less severe course of disease can be observed in patients younger than fourteen years of age. They show primarily meningeal courses and less likely meningoencephalitic or meningoencephalomyelitic courses (Kunze et al 2004 and 2005, Kaiser 1999, Kaiser 2000, Noack 1997, Harasek 1974) though there are reports of severe cases (Logar et al 2000, Messner 1979, Helwig et al 1983, Falk et al 1979). It is possible that disease severity depends on the respective virus strain, on the humoral response of the immune system of the afflicted person and whether the virus is able to invade the CNS (Dörrbecker et al 2010), with these factors being non-mutually exclusive as mechanisms determining disease outcome.

## 3.2. TBE in domestic animals

TBE is known to occasionally cause clinical symptoms in dogs and horses. The clinical symptoms in the aforementioned animals closely resemble those in infected humans (Pfeffer and Leschnik 2018), although severe neurological symptoms are rare in horses (Cavalleri 2017). With the low number of case reports and horses with clinical symptoms prohibiting valid estimation of the impact of TBE on horse health (Luckschander et al 1999, Müller et al 2006, Klaus et al 2013). Due to their weight and the general problems linked to inability to stand for horses, the prognosis for horses is more unfavourable in severe cases than for dogs, which can be treated much better if their musculoskeletal system is affected. Nonetheless, the fatal outcome in dogs is reported to range from 16-50% of clinical cases (Klimeš et al 2001, Leschnik et al 2002, Leschnik et al 2008).

Since horses are very unlikely to show clinical signs and are – in most cases – relatively confined in their movement, they are discussed as possible sentinel animals, whose serum virus titre could help to determine whether pathogenic TBE strains can be found in their surroundings (Pfeffer and Leschnik 2018). The seropositivity of horses living in natural foci is on average 24% (Cavalleri 2017). One study discovered an infection rate of 26,1% for horses of the same breed in Austria, with a higher likelihood for male horses to be seropositive (Rushton et al 2013).

TBE in cattle and small ruminants is mostly subclinical, but alimentary infections to humans via their milk gives weight to the infection of these animals (Holzmann et al 2009). Nonetheless, in rare cases, clinically apparent disease may occur in ruminants (Bagó et al 2002), with symptoms similar to those of humans.

# III. Paper

## 1. First publication: Continuous isolation of Tick-borne encephalitis virus from adult *Dermacentor reticulatus* ticks in an endemic area in Germany

The following publication describes the occurrence of TBEV in another tick species than *Ixodes ricinus*, namely *Dermacentor reticulatus*. This focus was found in Saxony and showed that other tick species were able to transmit the virus and uphold a natural focus.

It was published on March, 12th 2019 in Parasites & Vectors 12, Article number: 90 (2019). https://doi.org/10.1186/s13071-019-3346-6

**Continuous isolation of Tick-borne encephalitis virus from adult *Dermacentor reticulatus* ticks in an endemic area in Germany**

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**Abstract**

**Background:** The tick-borne encephalitis (TBE) virus is transmitted to humans and animals through tick bites and is thought to circulate in very strict outlined natural environments called natural foci. The most common tick serving as vector for TBE virus in Central Europe is *Ixodes ricinus*, rarely it is found in other tick species and, so far, only in Poland in *Dermacentor reticulatus* tick. Here we describe a new TBE focus in the district of northern Saxony, Germany, outside the known risk areas as defined by the national Robert-Koch-Institute.

**Methods:**

**Findings:** Between autumn 2016 and spring 2018 TBE virus was detected eleven times in flagged adults *D. reticulatus* (n=1,534), while *I. ricinus* nymphs (n=349) tested positive for TBE virus only once. *I. ricinus* males (n=33) and females (n=30), as well as five *I. inopinatus* (2 females, 3 males) and 14 *Haemaphysalis concinna* (3 females, 11 nymphs) tested negative for TBE virus by means of real-time RT-PCR. TBE virus was not detected in *I. ricinus* during the summer, when *D. reticulatus* was not active. Sequence comparison of the entire E gene of the isolated virus strains resembled each other with only 3 nucleotide differences. The most closely related viral sequences belong to TBE virus strains from Poland and Neustadt an der Waldnaab approximately 200 km east and 200 km south-west of the new focus.

**Conclusions:** This is the first report of a TBE virus circulation in an endemic region where *D. reticulatus* and *I. ricinus* do occur sympatrically in nature but where *D. reticulatus* seems to play a key role in virus circulation.

**Keywords:** *Dermacentor reticulatus*, TBE virus, Saxony, Germany

**Background**

The tick-borne encephalitis (TBE) virus (genus *Flavivirus,* family *Flaviviridae*) is the etiological agent of TBE, the medically most important member of the tick-borne serocomplex group (Casals, 1957; Gould and Solomon, 2008).

At present, three subtypes of TBE virus – the European (western) subtype (TBEV-EU), the Sibirian subtype (TBEV-Sib) and the Far-Eastern subtype (TBEV-FE) – are recognized (Ecker

et al., 1999). Russian virologists have claimed two new subtypes, both isolated in the Lake Baikal region in Siberia which are genetically more distant to each of the three currently accepted TBE virus subtypes (Demina et al., 2010).

Of the 52 species of ixodid tick known from the western Palearctic (Estrada-Peña et al., 2018), eight species from three genera are known to be able to transmit TBE virus, and the virus has been isolated from at least 14 other species (Chitimia-Dobler et al., 2018, Table 1). *Ixodes ricinus*, the most commonly encountered European tick species, is considered to be the major vector of the European TBE virus (Süss, 2003). Lichard and Kozuch (1967) were able to show TBE virus persistence and transmission to white mice by *Ixodes arboricola*, which is considered to be a secondary amplifying vector of TBE virus in nature (Grešiková 1972). *Ixodes persulcatus* is the main vector tick species known to transmit TBEV-Sib and TBEV-FE (Nuttall and Labuda, 1994; Labuda and Nuttall, 2004). *Haemaphysalis concinna* is a known vector of TBE virus as well (Kožuch and Nosek, 1980; Khazova and Iastrebov, 2001). Experimental proof for the vector competence of *Haemaphysalis inermis* is available for TBE virus in Nosek et al. (1972). *Ixodes gibbosus* is a marginal vector in the Mediterranean (Hubálek and Rudolf, 2012). In addition, TBE virus has been found in numerous other tick species, but transmission has not been demonstrated, for example in *Ixodes frontalis* (Labuda and Nuttall, 2004; Hillyard, 1996; Obsomer et al., 2013). The virus has been isolated in the Czech Republic from female and nymphal *I. hexagonus* infesting a hedgehog (Krivanec et al., 1988), as well as in Croatia from a pool of three females removed from a red fox (Jemeršić et al., 2014). *Haemaphysalis punctata* also has been associated with TBE virus (Hubálek et al., 1989).

The genus *Dermacentor* (family Ixodidae) includes 35 species and has a worldwide distribution, except for Australia (Guglielmone et al., 2014). *Dermacentor* species are found mostly in Europe, Asia, and North America (Nicholson et al., 2009; Sonenshine et al., 2002). In Europe, two species, *Dermacentor reticulatus* (‘the ornate dog tick’), *Dermacentor marginatus* (‘the ornate sheep tick’), and in Asia, *Dermacentor nuttalli*, are all associated with TBE virus. Both, *D. marginatus* and *D. reticulatus* are competent vectors of this virus (Hoogstraal, 1966; Kožuch and Nosek, 1971; Nosek, 1972). The role of *Dermacentor* ticks in the circulation of TBE virus in the environment however, is unclear and poorly studied (Karbowiak, 2014; Karbowiak and Kiewra 2010). *D. reticulatus* appears to be spreading, with distribution area and population density that have been increasing during recent decades (Karbowiak, 2014; Karbowiak and Kiewra 2010; Dautel et al., 2006; Chitimia-Dobler, 2015; Rubel et al., 2016). In eastern Poland, the average prevalence of infection with TBE virus found in *D. reticulatus* was 10.8%; this is considerably higher than the prevalence found in *I. ricinus* (1.6%) (Wojcik-Fatla et al., 2011). Prevalence in *D. reticulatus* ticks from Białowieża Primeval Forest was similar (1.58%) (Biernat et al., 2014) to that of *I. ricinus* (1.30%) (Biernat

et al., 2016), as was the case in Moldova (*I. ricinus* 3.8%, *D. reticulatus* 3.9%, but *Haemaphysalis punctata* 8.8%) (Ponomareva et al., 2015). However, the dynamics of TBE virus in the *D. reticulatus* tick population in TBE natural foci has not been studied, so far, and no data on TBE virus in *D. reticulatus* in Central Europe are available.

Every active tick stage can be infected with TBE virus (Karbowiak and Biernat, 2016), and approximately 0.1-5% of the ticks in an endemic area carry the virus (Süss, 2003). The female *D. reticulatus* tick is considered to play a major role in virus transmission while in *I. ricinus* the nymph is the key stage in viral transmission. Tick males, which either do not feed or feed for only a short time, might also be involved in virus transmission and appear to be responsible for the asymptomatic infections and further may contribute to host immunity (Korenberg et al., 1986). TBE virus invades all tick tissues, including the salivary glands and ovaries (Karbowiak and Biernat, 2016), thus it may be transmitted in various ways: 1) take up the virus during viremia of the host, 2) via co-feeding (direct uptake of the virus from one tick feeding in close proximity of a tick harboring the virus without the need of a viremia of the host both ticks feed on, 3) transstadial, i.e. from one life stage to the next life stage after molting 4) transovarial (vertical from female tick to her eggs) and 5) transsexual which means between male and female during mating on the host to 6) infect the next host via saliva, (Filippova, 1985; Labuda and Randolph, 1999; Rosa et al., 2003; Satz, 2006).

In this study we describe a TBE natural focus in Germany and the phenology of TBE virus in *D. reticulatus* for a period of three seasons.

## Materials and Methods

### Study site

In July 2016, the first human TBE case ever was reported from the district northern Saxony in the German Federal State of Saxony, a so far TBE non-endemic area. The exact location where the infection most likely took place could be identified with the patient's information. The landscape is dominated by a rather young mixed forest consisting of birch, oak, maple and pine trees, the latter up to 80 years old, while the deciduous trees are mostly not older than 20 years of age. In the undergrowth blueberries, blackberries and raspberries can be found. This forest is interrupted by patches of meadow and surrounded by larger stretches of agricultural land. The next human settlement is a small village (Battaune), which is about 500 metres away; no industrial areas or waste disposal sites are known as a part of this area. Only a small area surrounding a torn down house is used as illegal dump site for household trash. There are eleven rather large areas of life stock holding within a radius of approximately one kilometre of the flagging area.

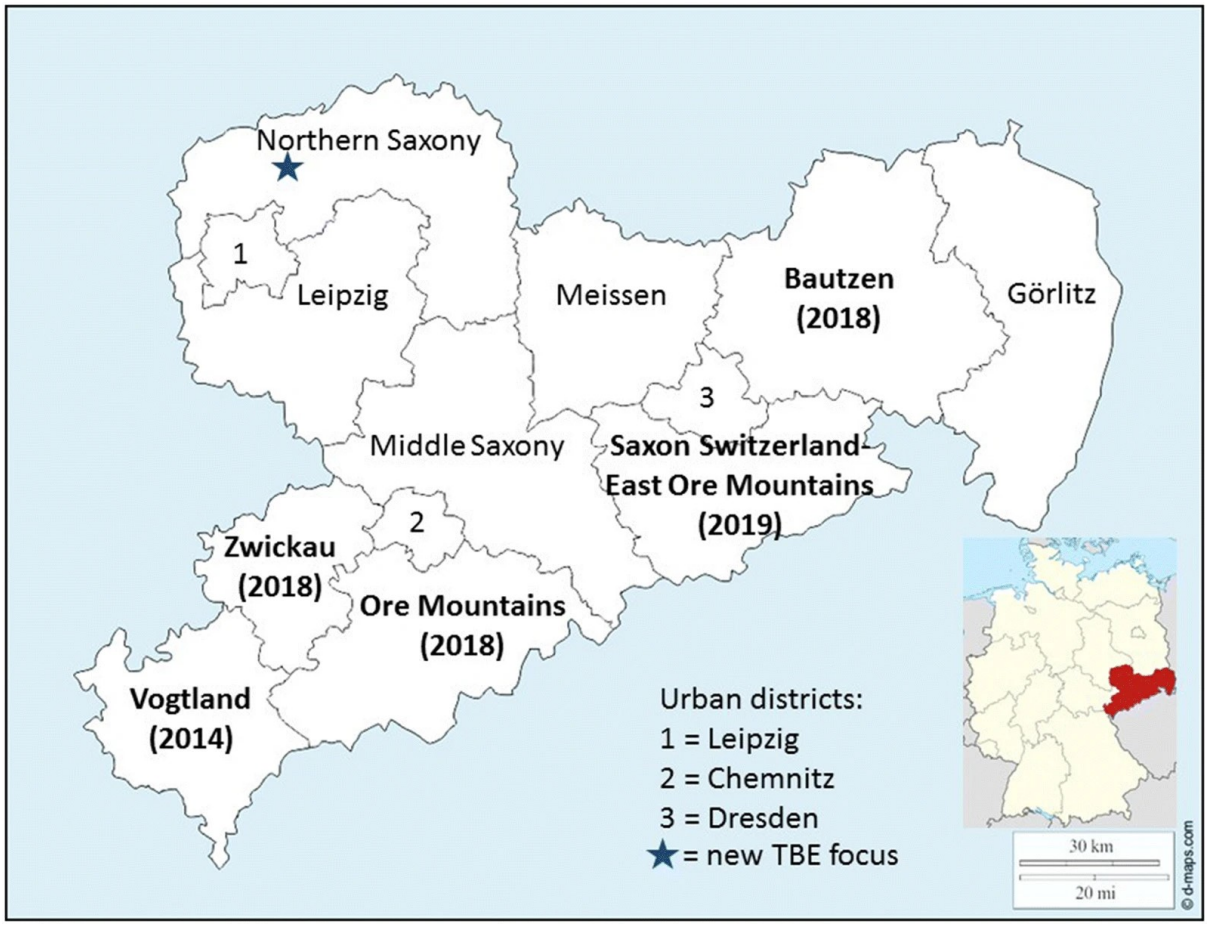


**Fig. 1:** A map of Saxony showing all counties. Names of counties considered as TBE risk area according to the federal Robert Koch Institute are named with bold lettering showing the year since they were considered TBE risk area in parentheses. The new TBE focus described here is indicated by an asterisk. At the time of our study, it was about 150 km north of the next known risk area

### Tick collection and identification

All specimens were collected as questing ticks by flagging as part of a tick-borne encephalitis (TBE) program in Battaune, Federal State of Saxony, Germany, at the end of September 2016, in February, April, July and September 2017, and April 2018. The ticks were kept alive in 50 ml plastic tubes until identification and further testing in the laboratory. Ticks were identified to the species level using the morphological characters according to Feider (1965), Filippova (1977) and Estrada-Peña et al. (2014).

### Nucleic acid extraction and PCR

Ticks were processed in pools as follows: Ten nymphs or five adults (females and males separated) per pool. Ticks were homogenized using 1 ml Minimum Essential Medium (MEM, Invitrogen, Karlsruhe, Germany) containing an antibiotic-antimycotic solution (Invitrogen, Karlsruhe, Germany) using the Fast Prep Savant FP120 tissue lyser (Bio101, Vista, USA), with three rounds at speed 6.5 m/s, for 30 seconds each. Total nucleic acid was extracted using the MagNA Pure LC RNA/DNA Kit (Roche, Mannheim, Germany) in a MagNA Pure LC instrument (Roche, Mannheim, Germany) according to the manufacturer's instructions. The total nucleic acid was extracted in 50 µl and a 5 µl aliquot was tested for TBE virus RNA using a real-time RT-PCR (Schwaiger and Cassinotti, 2003).

**Sequence analyses of TBE virus E-gene**

The E-gene was amplified with a conventional PCR directly from positive tick nucleic acid extractions. The products were purified after gel electrophoresis and processed as described (Frey et al., 2012). The purified PCR products of about 1,600 nt in lengths were sequenced by GATC Biotech (Eurofins Genomics, Ebersberg, Germany). De novo Assembly of the three Chromatogram files per sample was performed using the Assembler of geneious 9.1.5 (http://www.geneious.com, Kearse et al., 2012). For analysis the sequences were cut to 1,488 nt, the exact length of the E-gene sequence. The Multi Sequence Alignment was performed using the ClustalW algorithm (Thompson et al., 1994), also embedded in geneious 9.1.5. Phylogenetic analysis was conducted using the MEGA 6.0 software (http:/www.megasoftware.net, Tamura et al., 2013). A maximum likelihood tree with selected sequences was generated, using the Tamura-Nei substitution-model and 1000 bootstraps for phylogeny testing.

For all PCR methods, standard procedures for PCR testing (three room concept, inclusion of positive and negative controls, extraction controls) were included in each run.

**Results**

In September 2016, ticks were collected during three consecutive days. In total, 996 ticks were collected: 816 *D. reticulatus* (502 females and 314 males); 114 *I. ricinus* (12 females, 12 males, 91 nymphs, 58 larvae); four *I. inopinatus* (two females and two males) and three *H. concinna* nymphs. Totally, 174 pools were tested, with four positive pools, three *D. reticulatus* pools (one female and two males) and one *I. ricinus* nymph pool.

In 2017, 863 ticks were collected all over the year, from which 620 *D. reticulatus* (266 males and 354 females), 231 *I. ricinus* (18 males, 10 females and 203 nymphs), *I. inopinatus* (one male), and 11 *H. concinna* (three females and eight nymphs). Details regarding tick species activity are presented in Table 1 and Figure 1. In February, 90 pools were tested, three *D.*

*reticulatus* female pools were positive. In April, 16 pools were tested; 12 *D. reticulatus* pools and four *I. ricinus* pools, with one *D. reticulatus* female positive pool. In July, 27 pools were analysed: 22 *I. ricinus* pools and five *H. concinna* pools, none of them were positive. In September, 38 pools were investigated and two pools were positive, one *D. reticulatus* female pool and one *I. ricinus* nymph pool.

In 2018, 164 ticks were collected, 98 *D. reticulatus* (41 males and 57 females) and 66 *I. ricinus* (three males, eight females and 55 nymphs). A total of 33 pools were tested and one male *D. reticulatus* pool was positive.

TBE virus was detected and isolated from three male and six female *D. reticulatus* pools and two *I. ricinus* nymph's pools.

The minimal infection rate (MIR) for *D. reticulatus* varied between 0.36% in September 2016; 0.74% in February, 1.69% in April and 0.63% in September 2017; 0.65% in April 2018. MIR for *I. ricinus* was 1% in September 2016 and 4% in September 2017.

A total of 11 E-genes sequences of the positive tick pools were generated and analysed. The analysis of the E-genes of the TBE virus showed a close relationship to TBE E-gene sequences from Poland, Southern Germany and Switzerland. A phylogenetic tree is shown in Figure 2.

| | *Dermacentor reticulatus* | | *Ixodes ricinus* | | | | *Ixodes inopinatus* | | *Haemaphysalis concinna* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | females | males | females | males | nymphs | larvae | females | males | females | nymphs |
| September 2016 | 502(1) | 314(2) | 12 | 12 | 91(1) | 58 | 2 | 2 | 0 | 3 |
| February 2017 | 234(3) | 169 | 3 | 2 | 7 | 0 | 0 | 1 | 0 | 0 |
| April 2017 | 31(1) | 28 | 1 | 4 | 22 | 0 | 0 | 0 | 0 | 0 |
| July 2017 | 0 | 0 | 6 | 10 | 150 | 0 | 0 | 0 | 3 | 7 |
| September 2017 | 89(1) | 69 | 0 | 2 | 24(1) | 0 | 0 | 0 | 0 | 1 |
| April 2018 | 57(1) | 41 | 8 | 3 | 55 | 0 | 0 | 0 | 0 | 0 |
| Total | 913(7) | 621(2) | 30 | 33 | 349(2) | 58 | 2 | 3 | 3 | 11 |

**Table 1:** Tick species and stages collected during the study and the positive pools

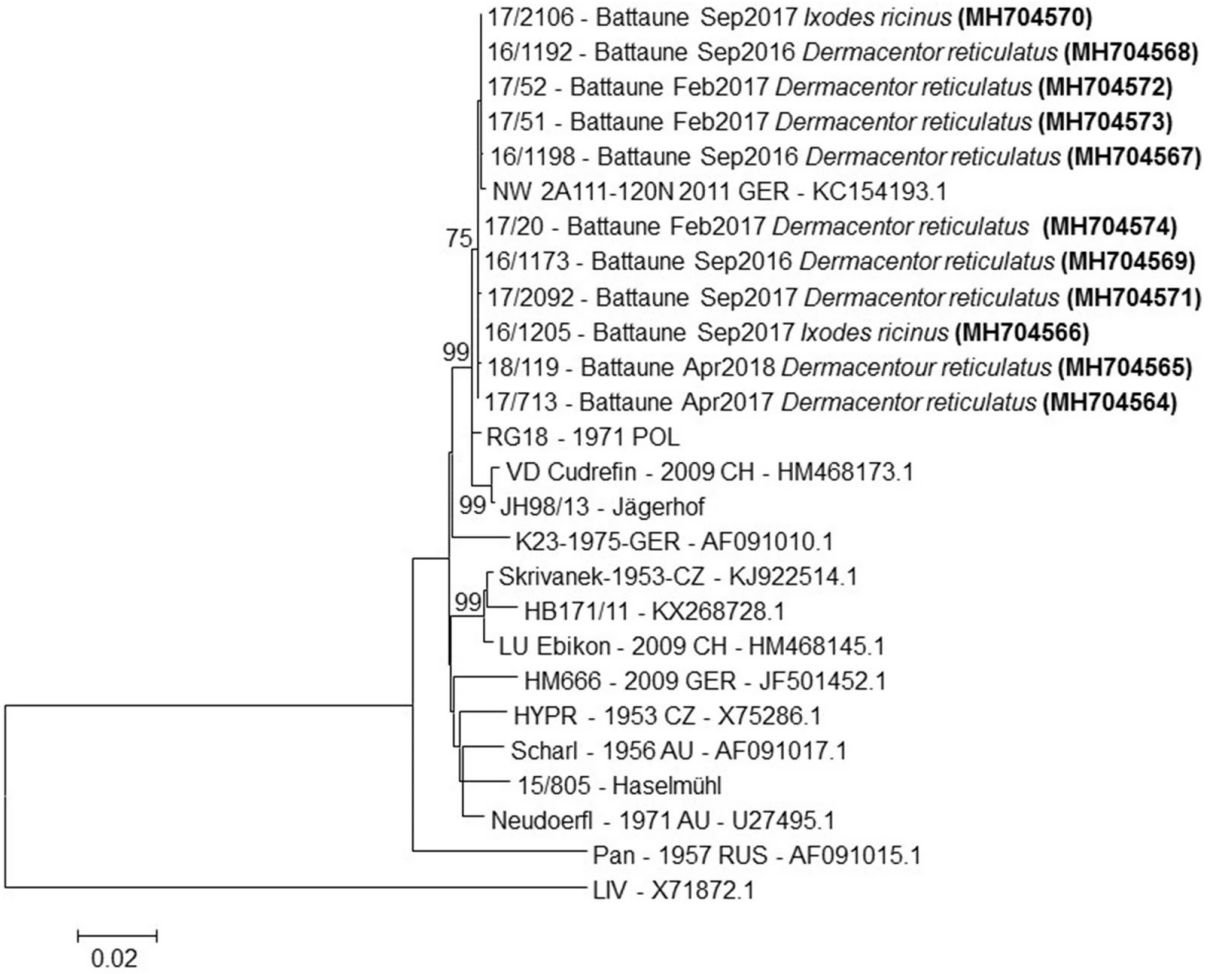


**Fig. 2**: Phylogenetic analysis of complete E gene sequences of European TBE virus strains using louping ill virus (LIV) as outgroup. Nucleotide sequences generated for this manuscript are given in bold and the name of the respective tick species is provided. The countries of origin are given as: GER, Germany; CZ, Czech Republic; AU, Austria; CH, Switzerland; POL, Poland; RUS, Russia. GenBank accession numbers are also provided

**Discussion**

TBE virus was continuously found in *D. reticulatus* during three sampling seasons in an area of sympatric occurrence with *I. ricinus* and to a lesser extent *I. inopinatus* and *Haemaphysalis concinna*. Although TBE virus has previously been detected in *D. reticulatus* ticks (Wojcik-Fatla et al. 2011, Biernat et al 2016) and its capabilities to transmit TBE virus has been demonstrated (Kožuch and Nosek 1971), this was a surprising finding. Maintenance of TBE virus by *D. reticulatus* in natural foci in the absence of *Ixodes* ticks was considered very doubtful [Katagina et al 2013], but was supposed to possibly support TBE virus circulation in *I. ricinus* populations. Our results suggest the opposite, i.e., that *D. reticulatus* is the main actor in the maintenance of TBE virus in this natural focus, as we found it throughout our sampling period and during the summer when adult *D. reticulatus* cannot be flagged, the virus was also

absent in the *I. ricinus* population. Sympatric occurrence of *D. reticulatus* with *Ixodes persulcatus*, a sibling species of *I. ricinus* abundant throughout Central Asia, found the virus in both tick species (Kislenko et al., 1987). In contrast to the situation described here, the authors found a sympatric occurrence of *D. reticulatus* and *I. persulcatus* throughout the year while in Germany *D. reticulatus* adults cannot be collected by flagging in June, July and August (Obiegala et al., 2017?). This might be the difference explaining the higher prevalence found in our study. Recent reports from Poland support our findings. Here, in 10.8% of 148 *D. reticulatus* and in only 1.6% of 875 *I. ricinus* TBE virus was detected (Wojcik-Fatla et al. 2011). As immature stages of all these tick species share their hosts, infection may occur on viremic rodents or during co-feeding on the same small mammal as demonstrated in Udmurtia (Kislanko et al., 1987). As transstadial transmission was also previously demonstrated for *D. reticulatus* (Karbowiak et al., 2016), the most likely source of the TBE virus infection of immatures of this nidiculous tick species is the rodent, known to serve a reservoir host for TBE virus (Nuttall and Labuda, 1994). Future attempts have to investigate the role of small mammals in the TBE virus transmission cycle at this natural focus. However, detection and isolation of TBE virus in *Dermacentor* tick species is not very common and the reasons for this is not known or not understood. The repeated findings of TBE virus positive *D. reticulatus* ticks in three consecutive years, 2016-2018, are noticeable. It may be speculated that the fact that TBE virus obviously was recently introduced into this area (as no human TBE cases were notified since TBE became a notifiable disease in Germany in 2001) may be the reason that the most abundant tick species became the main carrier of the virus. At least our phylogenetic analyses did not find a tricking difference between nucleotide sequences derived for either *D. reticulatus* or *I. ricinus* ticks. The branching of our sequences together with a sequence for Poland (approximately 200 km east) and Neustadt an der Waldnaab (approximately 200 km south-west) are in line with a general east to west evolution of TBE viruses in general (Weidmann et al. 2013) but they do not provide hints why *D. reticulatus* seem to be the key in TBE virus maintenance here.

It is interesting to note that positive *I. ricinus* nymphs were found only in September, when both *I. ricinus* and *D. reticulatus* activity was high. For more information, if this was an accidental occurrence more studies should be conducted. However, the positive testing of TBE virus might possibility support the theory that the tick species *D. reticulatus* plays a major part in the conservation of this TBE natural focus. This would be conclusive with the findings of the Polish research team of Biernat et al. (2014) in Poland.

**List of abbreviations**

Not applicable

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**Author contributions**

LCD, GD and planned and organized the study. LCD, GD, MP, NK, and MB collected ticks. LCD performed species identification of ticks. LCD and GL extracted DNA. MB tested for TBE virus and performed the E genes analysis. LCD and MP wrote the manuscript with input from GD, MB and GL. All authors read and approved the final version.

**Competing interests**

The authors declare that they have no competing interests

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# 2. Second publication: Comparison of whole genomes of tick-borne encephalitis virus from mountainous alpine regions and regions with a lower altitude

The second publication compares TBE viruses from alpine regions to regions on a lower altitude. This publication shows that we could find no evidence of common genetic factors or a common virus ancestor of the viruses in our different alpine regions.

It was first published on January 24th 2021 in Virus Genes 57, pages 217–221 (2021). https://doi.org/10.1007/s11262-020-01821-w

# Comparison of whole genomes of tick-borne encephalitis virus from mountainous alpine regions and regions with a lower altitude

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**Abstract**

Tick-borne encephalitis (TBE) has been a notifiable disease in Germany since 2001. Its causative agent, the tick-borne encephalitis virus (TBEV), is the most important arbovirus in Europe and Northern Asia. The illness, caused by the European Subtype usually displays flu-like symptoms, but can result in sequelae and, in two percent of all cases, in death. Over the last few decades, the virus has spread into new habitats, such as higher altitudes in the Alpine region. For this study, it was hypothesized that the environmental challenges that the virus

might be exposed to at such altitudes could lead to the selection of viral strains with a higher resilience to such environmental factors. In order to determine whether strains identified at higher altitudes possessed different genetic traits compared to viruses from lower altitudes, an analysis of viral genomes from higher Alpine altitudes (>500m above sea level) (n=5) and lower altitudes (<500m above sea level) (n=4) was performed. No common phylogenetic ancestry or shared amino acid substitutions could be identified that differentiated the alpine from the lowland viral strains. These findings support the idea of many individual introductions of TBEV into the alpine region and the establishment of foci due to non-viral specific factors like favorable conditions for vector species and host animals due to climate change.

**Keywords:** tick-borne encephalitis virus, mountains, genetic analysis, tick-borne encephalitis, tick-borne encephalitis virus strains

## Introduction

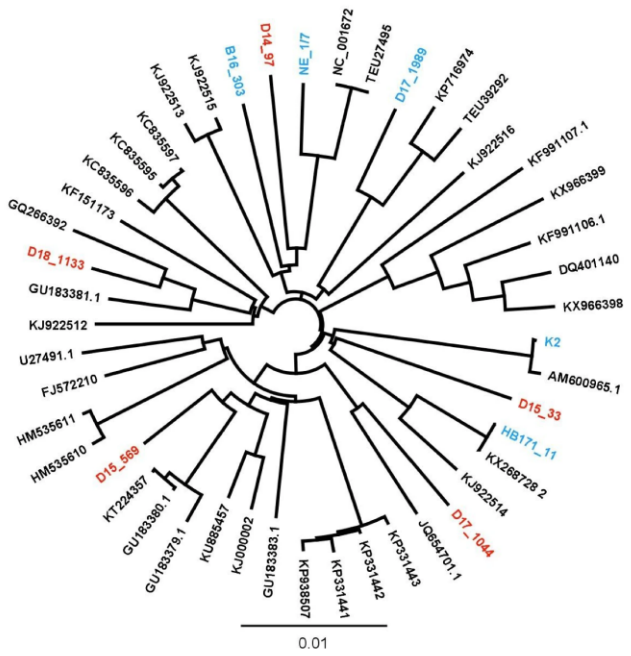
TBEV is the agent of tick-borne encephalitis (TBE), a severe infection of the central nervous system that may result in sequelae and can possibly end in disability or death. Currently, the virus is divided into three subtypes, the European, the Siberian and the Far Eastern subtype (Chumakov 1944, Pogodina 1981, Heinz et al 2000). TBEV is mostly transmitted via tick bites. However, especially in Eastern Europe, outbreaks caused by alimentary transmission through unpasteurized milk and soft cheese have been reported (Gritsun et al 2003, Moritsch and Kovac 1962, Pogodina 1958, Pogodina 1960).

In recent years the European subtype of TBEV has come more into focus since its distribution pattern changed significantly. The virus appeared in mountainous regions of the Alps previously considered free of natural foci of TBEV (Heinz et al 2015, RKI 2020). It is unclear, whether certain/specific genetic traits of the virus are responsible for this sudden claim of new endemic areas or if the climatic conditions changed and became more suitable for the natural transmission cycle. It is possible that the mountainous strains were naturally selected due to some small nucleotide polymorphisms (SNPs) in their genome that made them more resistant to the alpine environment. To shed light on the question, 5 different virus strains, isolated from these new endemic areas, were thoroughly analysed regarding their genomic sequences and compared to strains isolated from long established foci in lower altitudes.

## Results and Discussion

For all TBEV-EU strains included in the study the whole genome sequences were generated. The phylogenetic tree based on the nucleotide sequences showed no evidence of a common origin of the mountainous strains (**Figure 1**). The virus strains D15_33, K2 and HB171_11 had the closest phylogenetic relation to each other, despite coming from different elevation levels and collecting sites being 200 km apart.

(A)




(B)

| Strain | Location | Country | Year of Isolation | GPS - X | GPS- Y | Elevation | Sequencing material |
|---|---|---|---|---|---|---|---|
| D14_97 | Aschau | A | 2014 | 47,2583 | 11,8871 | 710 m | tick homogenate |
| D15_33 | Egelsee | A | 2015 | 47,6049 | 12,1707 | 563 m | tick homogenate |
| D15_569 | Wald | A | 2015 | 47,0778 | 10,8329 | 1364 m | tick homogenate |
| D17_1044 | Mühlau | D | 2017 | 47,7254 | 12,3938 | 611 m | tick homogenate |
| D18_1133 | Tres | I | 2018 | 46,3173 | 11,1069 | 858 m | tick homogenate |
| D17_1989 | Petting | D | 2017 | 47,9259 | 12,819 | 459 m | tick homogenate |
| BaWa16_303 | Haselmühl | D | 2016 | 49,409 | 11,8831 | 430 m | tick homogenate |
| NE_1/7 | Neudoerfl | A | 1975 | 47,7887 | 16,3051 | 266 m | babymouse brain |
| K2 | Karlsruhe | D | 1980 | 49,032 | 8,4136 | 116 m | babymouse brain |
| HB171_11 | Heselbach | D | 2011 | 49,2973 | 12,2006 | 432 m | VeroB4 P0/P1 |

**Figure 1: (A)** Phylogenetic tree of whole genomes of several TBEV-Eu strains. The whole genomes were amplified in three DNA amplicons covering the whole genome (Andersen et al. 2019). For sequencing the Illumina MiSeq platform and the MiSeq reagent kit V3 (Illumina, Inc., San Diego, USA) was used, following the manufacturer's instructions. Assembly was performed using the software Spades v.3.12. For the phylogenetic comparison, available TBEV-Eu whole genome sequences published in the NCBI GenBank database were chosen. The tree was generated using the maximum likelihood approach and 1000 bootstraps were implemented for statistical support. The strains used in this study are highlighted in red (alpine regions) and blue (lower altitudes). **(B)** Table containing the meta data for the TBEV strains used in the analysis.

In **Table 1** the different strains have been compared in regard to nucleotide changes/ amino acid differences. This analysis reveals that the strain K2 differs the most from strain NE_1/7 (Neudoerfl) with 219 nucleotide changes (shown in italics), whereas the strains D15_569 and D17_1044 show the fewest differences (shown in bold). Regarding amino acid substitutions the biggest difference of 40 amino acid changes is between strains K2 and D17_1989 (highlighted in italics) and the least amino acid difference can be observed between the strains HB171_11 and BaWa16_303 with only 19 amino acid changes (indicated in bold).

| | **D14_97** | **D15_33** | **D15_569** | **D17_1044** | **D18_1133** | **D17_1989** | **BaWa16_303** | **NE_1/7** | **K2** | **HB171_11** |
|---|---|---|---|---|---|---|---|---|---|---|
| **D14_97** | | 97,335 (273) | 97,648 (241) | 97,326 (274) | 97,55 (251) | 97,374 (269) | 97,696 (236) | 97,804 (225) | 97,306 (276) | 97,365 (270) |
| **D15_33** | 99,268 (25) | | 97,726 (233) | 97,413 (265) | 97,599 (246) | 97,443 (262) | 97,384 (268) | 97,394 (267) | 97,433 (263) | 97,628 (243) |
| **D15_569** | 99,18 (28) | 99,268 (25) | | **97,921 (213)** | 97,833 (222) | 97,657 (240) | 97,706 (235) | 97,667 (239) | 97,677 (238) | 97,862 (219) |
| **D17_1044** | 99,18 (28) | 99,209 (27) | 99,121 (30) | | 97,521 (254) | 97,433 (263) | 97,365 (270) | 97,365 (270) | 97,345 (272) | 97,531 (253) |
| **D18_1133** | 99,356 (22) | 99,356 (22) | 99,297 (24) | 99,238 (26) | | 97,531 (253) | 97,56 (250) | 97,501 (256) | 97,54 (252) | 97,589 (247) |
| **D17_1989** | 99,092 (31) | 99,151 (29) | 99,063 (32) | 99,151 (29) | 99,092 (31) | | 97,579 (248) | 97,462 (260) | 97,267 (280) | 97,482 (258) |
| **BaWa16_303** | 99,385 (21) | 99,356 (22) | 99,268 (25) | 99,268 (25) | 99,385 (21) | 99,151 (29) | | 97,823 (223) | 97,355 (271) | 97,511 (255) |
| **NE_1/7** | 99,238 (26) | 99,209 (27) | 99,121 (30) | 99,238 (26) | 99,238 (26) | 99,033 (33) | 99,385 (21) | | *97,257 (281)* | 97,384 (268) |
| **K2** | 99,092 (31) | 99,121 (30) | 99,033 (33) | 99,033 (33) | 99,121 (30) | *98,828 (40)* | 99,238 (26) | 99,033 (33) | | 97,609 (245) |
| **HB171_11** | 99,297 (24) | 99,326 (23) | 99,18 (28) | 99,18 (28) | 99,297 (24) | 99,063 (32) | **99,443 (19)** | 99,238 (26) | 99,151 (29) | |

**Table 1:** Comparison of nucleotide changes to resulting amino acid changes of all nine virus strains. The upper right part of the table shows the nucleotide identities given in percentage and the absolute number of snps in brackets. The analysis was performed by the software Geneious. The lower left part shows the identical projections on amino acid level. For analysis

the whole open reading frame was used. The biggest difference is shown in *italics*, while the strain combination with the least difference is shown in **bold**.

All ten amino acid sequences were aligned and analysed for substitutions. No common amino acid substitution differentiating the alpine and the lowland strains was found. All virus proteins were analysed separately. The capsid protein showed up to two individual changes in the amino acid sequence. The prM/M protein showed up to two individual changes in the sequence with only strain HB171_11 having a non-synonymous change of T141I. The strain D14_97 showed four amino acid changes in the E-gene sequence, two of which were heterologous and may affect the superficial charge of the virus membrane. Other non-synonymous changes were found in strain D15_569 with L459S, strain D17_1989 that has a Y130H and K2 with A83T. Y130H has already been correlated with increased neuroinvasiveness in immunodeficient mice (Pletnev 2001).

The NS1 protein showed up to two individual amino acid changes. Strain D17_1044 showed a non-synonymous substitution of the non-polar I127T, K2 one of P103S and HB171_11 A41T. Compared to its length the most variable protein was NS2A. The three strains D15_33, K2 and HB171_11 showed the same synonymous substitution of V41I and the strains D17_1044 and D17_1989 shared a substitution of I53M. The latter is remarkable, since both belong to two different genetic clusters and share another substitution in their NS3 protein. Within the NS2B amino acid alignment two non-synonymous changes could be identified for the strains D15_569 and HB171_11. For NS3 up to three individual substitutions were found and three of the strains (D15_33, D18_1133 and K2) showed non-synonymous changes. The NS4A showed one substitution in three viruses, with only the substitution of Gly100 for serine in strain D17_1044 being non-synonymous. Three strains (D17_1989, K2 and HB171_11) had up to two individual substitutions in the NS4B protein.

The NS5 showed up to fourteen amino acid substitutions ranging from one to seven individual changes. The virus with most individual changes was strain D17_1044, followed by strain D17_1989.

The virus strain with the fewest individual amino acid changes was strain D18_1133 with 23% changes in their amino acid sequence. It was followed by strain D14_97 with 33.3% individual changes. The virus strain with most individual changes was HB171_11 with 48%.

Therefore, a phylogenetic analysis often results in poor statistical support and has to be interpreted very cautiously. The comparison of our TBEV strains confirms this observation, since the genetic difference of the ten TBEV strains in our study was 0.028 substitutions per site in total. Over the last two decades a clear shift in the distribution of TBE virus from lower to higher altitudes has been observed. So far, the possible effects on the genomic characteristics of TBEV are still unclear. One working hypothesis was that the virus replication had to adapt to harsh environmental conditions. Therefore, these strains might exhibit certain specific changes in their genomes as a response to the conditions they were exposed in regions with higher altitudes. Another explanation is that every strain from a mountainous area is derived from a common ancestor adapted to the conditions found in mountains. After further distribution, they could form new natural foci. Furthermore, a change in climatic and therefore eco-epidemiological conditions in the higher altitudes might lead to better conditions for TBEV replication in ticks.

We were not able to find a common specific genetic trait shared by all strains from areas of an altitude above 500m above sea level. According to the phylogenetic analysis, each virus strain was probably introduced individually into its location and found favourable environmental conditions (possibly due to climate changes). We observed some of the amino acid changes described in the paper by Formanová et al 2014 in all eight virus strains (namely Ile167V, E127D, V201I and G206R). In addition, T33S was found in HB171_11 and D17_1989 and I53M in D17_1044 and D17_1989. Furthermore, we could not determine a close phylogenetic relationship between the mountainous strains. In fact, strain D15_33 was genetically most closely related to the lower altitudinal region strains HB171_11 and K2 in our analysis. This is another indicator for the newly emerged strains to be distributed into new areas by chance.

NS2A is said to be involved in the shift between RNA packaging and RNA replication (Khromykh et al 2001, Leung et al 2008), its high variability could be the reason for the different replication rates and infectivity of different TBEV strains. The same could apply to the high variability in NS2B, a protein suspected to be involved in modulating the membrane permeability during infection (Chang et al 1999). To confirm or disprove this hypothesis further experiments need to be undertaken.
Since we could not find any hints pointing towards specific genetic characteristics of TBEV strains from higher altitudes, we assume that other reasons for the new distributional pattern are responsible for the emerging alpine distribution of TBEV .

As shown by Rubel et. al in 2017 the Alps, have undergone a severe climatic shift since 1876. It can be assumed that this shift contributes to transformation of the Alpine environment and

therefore, the distribution of vector species, and host animals might change. This has yet to be proven by further research.

**Statement of author contributions**

The authors declare that they have no conflict of interest.

**Research involving Human Participants and/or Animals**

Not applicable

**Informed consent**

Not applicable

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# IV. Discussion

It has been observed in the Alps and Central Europe, that the main vector for TBE virus, *Ixodes ricinus*, has spread into higher altitudes than earlier studies. In the Czech Republic research has been published with an altitudinal limit for *I. ricinus* at 700 to 750 m above sea level over the country (Cerný et al 1965). Whereas, nearly 40 years later research resulted in findings of *I. ricinus* nymphs at 800 to 1300 m above sea level (Materna et al 2005, Materna et al 2008, Daniel et al 2003, Daniel et al 2005). Linked to the findings of I. ricinus in higher altitudes the occurrence of TBE virus foci higher above sea level were noted (Zeman and Beneš 2004, Heinz et al 2015). Additionally, to a broader distribution of TBE virus vectors we wished to examine whether the TBE virus shows adaptation to the conditions found in higher altitudes. Our examination showed no evidence for genetic virus adaptations to higher altitudes in our study, but as absence of evidence is not evidence of absence, we cannot completely exclude the possibility of such adaptive genetic changes within the TBE virus.

Considering the relationship of virus, vector and environment we would nonetheless argue that the reasons for the occurrence of TBE virus in higher altitudes should rather be related to the vector, the environment or both. Comparing Koeppen-Geiger maps of the last century to this century and considering calculations for the future, the most likely explanation seems to be climate changes, namely warming (Rubel et al 2017). Since a change in climate has an impact not only on temperature, but also on altitudinal zoonation, this could also affect vector distribution. In 2008 Materna et al were already able to show this in their surveillance of the occurrence of *I. ricinus* ticks in the Krkonoše mountains in the Czech Republic. Improved environmental conditions could have a positive impact on the population of host animals and their progeny and therefore, on the number of ticks. We could find no evidence of other tick species than *I. ricinus* or possibly *I. inopinatus* as vectors for TBE virus in the Alps, in contrast to a natural focus in Saxony, where the virus is apparent in *Dermacentor reticulatus* ticks found in three consecutive years.

Speaking against special adaptation to the Alpine environment or a common progenitor strain is a close relationship of alpine viruses to strains from different parts of Europe with highly differing altitudes. Furthermore, the Alpine strains do not differ in one unique regard from strains lower above sea level in viral growth curves. On the contrary, some differences in the strains occurred in some Alpine and non-Alpine strains simultaneously. There are strains that seem to be better adapted to cell culture, but no common pattern for Alpine strains could be found. Some show an overall low final virus titre, but one Alpine strain, strain “Muehlau”, shows

the highest titre of all examined strains. In further experiments, these tests on tick cell lines could help to prove or disprove the findings of this work.

After a close and thorough examination of our results, the most likely conclusion is that the spread into higher altitudes, as well as the shift of the natural focus spread, has its origin in climate warming. Since this, as stated earlier, has an effect on the vector and the host animals, it is the most likely explanation for the increased distribution of TBE. It can further cause change in human behaviour considering their activities, like hiking, but to prove this and how this possible change comes to pass, socio-epidemiological studies need to be conducted.

# V. Summary

Goal of this work was to characterise TBEV strains, the agent of TBE, in Alpine regions. TBE virus is a member of the family of *Flavivirus*, Genus *Flavivirus.* It is a single stranded virus of positive polarity.

We focused on testing whether these strains show certain qualities that are favourable for the environment found in Alpine regions, or are derived from a single Alpine progenitor strain, or if there is no connection at all. Since there has been a shift in the distribution of the virus to formerly naïve habitats, this was an important question for a possible prediction of further changes and to add to our understanding of the mechanisms driving the increased distribution of zoonotic diseases. Taking a multilateral approach, utilizing genetic analysis and kinetics, through growth curves, we investigated for Alpine-specific traits.

None of the experiments provided indications of Alpine virus genetic relationships or certain traits that all of these strains exhibit. More studies on tick cell lines could help to further investigate the drivers of virus strain manifestations in higher altitudes. The most likely explanation for the virus shift is changes in their vector or its distribution due to the effect climate warming has on the Alps and the surrounding Alpine regions. Since an ecological change in the Alps has already been demonstrated in earlier works and this is favourable for a habitat of the virus' vector, we deduced that climate change is the most likely cause of the observed change in cases reported.

# VI. Summary (German)

Das Ziel dieser Arbeit war es, verschiedene Stämme des europäischen Subtyps des Frühsommer-Meningo-Enzephalitis Virus (FSME Virus) zu charakterisieren. Bei dem FSME Virus handelt es sich um ein Virus aus der Familie der *Flaviviren*, Genus *Flavivirus*. Es ist ein einzelsträngiges, unbehülltes Virus mit positiver Polarität.

Der Fokus dieser Arbeit lag darauf, Bergstämme im Vergleich zu denen des Flachlandes zu charakterisieren. Dabei sollte herausgefunden werden, ob das Virus möglicherweise von einem alpinen Progenitor-Stamm abstammt, sich an die Umwelt in den Bergen angepasst hat oder ob es keinerlei Verbindung zwischen den alpinen Viren untereinander gibt. Diese Untersuchung erschien uns wichtig, da, unter anderem, einer Studie aus Österreich zufolge die Zahl der Erkrankungsfälle unter der ungeimpften Population an alt-etablierten Krankheitsherden kontinuierlich abnimmt, während in alpinen Regionen, die zuvor nicht mit Krankheitsfällen in Verbindung standen, vermehrt Infektionen auftreten. Es war uns wichtig, dies herauszufinden, um in Zukunft möglicherweise Prognosen über die weitere Entwicklung von zoonotischen Erkrankungen treffen zu können. Mit unserem multilateralen Ansatz von kinetischer Untersuchung und genetischer Analyse haben wir nach alpinspezifischen Viruseigenschaften gesucht.

Hierbei konnte kein Hinweis darauf gefunden werden, dass das erweiterte Verbreitungsgebiet über Veränderungen des Virus selbst zu erklären ist, sei es auf Grund eines alpinen Progenitorstammes oder genetischer Ähnlichkeiten, die dem Virus einen Vorteil in der Etablierung eines natürlichen Herdes bieten. Unsere Ergebnisse lassen den Schluss zu, dass es an einer größeren Verbreitung des Vektors, den Zecken, auf Grund veränderter Umweltfaktoren liegt, dass das Virus sich weiterverbreitet. Dadurch wäre es sinnvoll in zukünftigen Studien auf Zecken-Zellinien den Antrieb der Manifestationen von FSME in höher gelegenen Regionen weiter zu erforschen. Da eine ökologische Veränderung durch den Klimawandel unbestreitbar ist und auch das neue Habitate für Vektorzecken eröffnet, ist das als der wahrscheinlichste Grund für die veränderte Virusausbreitung und Fallzahlen anzunehmen.

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