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# Tick-borne encephalitis virus in the natural rodent reservoir: Experimental studies

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# Content

# 1. Introduction

Tick-borne encephalitis (TBE) was initially described in the early 1930s in the former USSR as an acute disease of the central nervous system (CNS) with a high death rate (Zlobin, Pogodina et al. 2017). A similar neurological disease was also diagnosed in Austria for the first time in an forest worker in 1931 (Schneider 1931). Around 1940, the first scientific report about the etiology of TBE and its causative agent, the tick-borne encephalitis virus (TBEV), reached the scientific world, describing a virus that showed a close resemblance to the Japanese encephalitis virus but was transmitted through ticks instead of mosquitos (Chumakov and Seitlenok 1940). Even in a very early report following the initial description rodents, especially hares and squirrels, are mentioned to be the so-called 'natural reservoirs' of the virus (Anonymous 1940). The basic idea of a reservoir host is an animal that develops a prolonged viremia that is potent enough to infect naive arthropod vectors, in the case of TBE hard ticks, without suffering negative losses in regards to longevity (WHO-position-paper 1967). As a result, the virus is spread among the tick population and could become endemic in a certain region (Pfeffer and Dobler 2010).

However, this explanation of the transmission cycle lacks actual scientific support and there is no unified definition of criteria that these so called 'reservoir hosts' have to fulfil (Kuno, Mackenzie et al. 2017). Discoveries like the non-viremic transmission of TBEV between ticks that feed on the same animal in close proximity, a process that is called "co-feeding" and can take place even when the host animal has already developed antibodies against the virus in question (Labuda, Kozuch et al. 1997), as well as the trans-stadial and trans-ovarial transmission of virus in the vector ticks themselves (Karbowiak and Biernat 2016), challenge this traditional view. In addition, there is a lack of studies performed in the suspected natural mammalian reservoir hosts, since most *in vivo* TBEV studies are using laboratory mice (Mandl 2005).

Bank voles (*Myodes glareolus*) and yellow-necked mice (*Apodemus flavicollis*) are often referred to as the natural small mammal reservoir hosts for TBEV (Süss 2003). At least in mainland Europe, this assumption is based on the results of numerous TBEV surveillance studies, where individuals of these two species are found with the highest prevalence and show the highest rates of both antibodies against TBEV and viral TBEV RNA detection

(Michelitsch, Wernike et al. 2019). In further parts of Eurasia, other small mammalians might take over their role, as it is discussed in the review article that is presented in this thesis (Michelitsch, Wernike et al. 2019). The second article in this thesis describes experimental infection studies that were carried out to assess the situation in mainland Europe (Michelitsch, Tews et al. 2019). For this, two distinct bank vole lineages were inoculated with different TBEV strains of the European subtype or with apathogenic Langatvirus (LGTV). Like TBEV, LGTV is a member of the tick-transmitted flavivirus group (Dobler 2010). Since LGTV is only endemic in Malaysia (Smith 1956), the virus was used as a reference for the interaction between bank voles and a virus that is not endemic in their distribution area. In comparison to that, the interaction between bank voles and local TBEV strains was studied.

# 2. Review of literature

To obtain an overview of the different animal species that are in close contact with TBEV in different parts of Eurasia and to assess the role of these potential reservoir hosts in the TBEV transmission cycle, a summarizing review article was published. This review is used as background information in this thesis to avoid repetition. Additional chapters on 'Tick borne encephalitis virus – taxonomy, and molecular characteristics', 'Tick borne encephalitis – the disease' and '*In vivo* studies in natural hosts' were added in the "review of the literature" part.

The reference section of the following manuscripts is presented in the style of the journal and are not included at the end of this document. The labeling of figures and tables corresponds to the published form of the manuscripts.

## 2.1. Tick borne encephalitis virus – taxonomy and molecular characteristics

TBEV is a member of the family *Flaviviridae* and therein belongs to the genus *Flavivirus* (Grard, Moureau et al. 2007). Most known members of this genus are vector-transmitted and many pose a serious threat to human health. Among the mosquito-transmitted flaviviruses, there are some representatives that caused recent outbreaks, like e.g. Zika virus (Cauchemez, Besnard et al. 2016, Ferguson, Cucunubá et al. 2016), West Nile virus (Ulbert 2019), Usutu virus (Roesch, Fajardo et al. 2019) and yellow fever virus (Shearer, Moyes et al. 2017). In contrast, tick-transmitted flaviviruses may not cause large-scale acute outbreak scenarios, due to the frequency of contact to humans, but can still have a tremendous impact on the lives of those who are affected (Holbrook 2017). Besides TBEV, LGTV, Omsk hemorrhagic fever virus, Powassan virus, Kyansanur forest disease virus and Alkhumra hemorrhagic fever virus are the most prominent members of the tick-transmitted flavivirus complex (Dobler 2010).

Based on the phylogenetic analysis of the surface glycoprotein E gene, TBEV is divided into three classical subtypes, the European (TBEV-Eu), the far eastern (TBEV-FE) and the Siberian (TBEV-Sib) one. (Ecker, Allison et al. 1999). Two additional, potential subtypes, the Baikalian (TBEV-Bkl) (Kovalev and Mukhacheva 2017) and the Himalayan (TBEV-Him) (Dai, Shang et al. 2018) were described only recently.

The structure of a mature virion is similar for all flaviviruses (Velay, Paz et al. 2019). It has the form of an enveloped, roughly spherical particle with a diameter of 50 nm. The envelope protein E gives shape to the virion in combination with the second membrane protein M (Kuhn, Zhang et al. 2002). This glycoprotein coat encases the positive oriented, single RNA strain that makes up the genome and lays in complex with the capsid (C) protein (Ma, Jones et al. 2004) (Figure 1A). The genome of TBEV has a length of approximately 11 kilobases and encodes for a single polyprotein (Lindenbach and Rice 2003). Co- and post-transcriptionally processes divide this polyprotein into three structural and seven non-structural proteins. The three structural proteins are the already mention proteins E, C as well as the precursor protein of the M protein (prM) (Pulkkinen, Butcher et al. 2018). During the maturation of TBEV virions, prM is cleaved by the enzyme furin shortly before the virion exists the cell (Stadler, Allison et al. 1997). The seven non-structural proteins are NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5

(Figure 1B). They perform several enzymatic functions in virus replication (Lindenbach and Rice 2003) and seemingly help modulate the hosts immune response (Best, Morris et al. 2005).

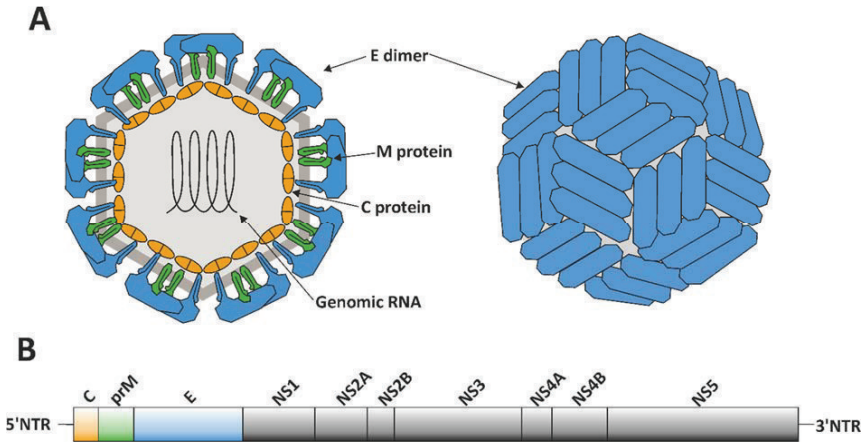


Figure 1: Schematic representation of the mature TBEV virion (A) and of the TBEV genome (B).

## 2.2. Tick borne encephalitis – the disease

TBE is the most important tick-transmitted, neurological disease in Eurasia. Humans acquire an infection predominantly through tick-bites (Dobler, Gniel et al. 2012). After an incubation period of 4 to 28 days, a first viremic phase takes place, spreading the virus systemically. During this first phase, unspecific symptoms like mild-fever, headache, myalgia, nausea and fatigue can occur. If the virus manages to cross the blood brain barrier (BBB) and penetrates the CNS, this first phase is followed by a second phase of disease where neurological symptoms start to appear. Between these two phases, an asymptomatic interval of one to two weeks can occur (Růžek, Avšič Županc et al. 2019).

Overall, 70% to 98% of human infections are supposedly asymptomatic, but since this assumption is based on retrospective epidemiological studies, mild cases with only flu-like symptoms may be overlooked (Bogovic and Strle 2015). Patients that are diagnosed with TBE show the typical biphasic course of infection in up to 46% of cases (Růžek, Avšič Županc et al. 2019), for infections with the European subtype of TBEV the estimated number is higher (75%) (Bogovic and Strle 2015). The second phase appears as either meningitis in about 50% of the adult patients, as meningoencephalitis in 40% and lastly in 10% as meningoencephalomyelitis or meningoencephaloradiculitis (Kaiser 1999, Du Four, Mertens et al. 2018). Patients that develop meningitis feel weak and sluggish, have stiff neck muscles and may experience headache, nausea and photophobia. For those suffering from meningoencephalitis, additional symptoms like delusions, hallucinations, epileptic seizures and a loss of orientation in place and time appear (Kaiser 2012). Patients developing a meningoencephalomyelitis experience flaccid paralysis of the limps, neck and back muscles. Even intercostal muscles and the diaphragm might be affected, resulting in respiratory failure (Růžek, Avšič Županc et al. 2019). Meningoencephaloradiculitis leads to damage of peripheral nerves, resulting in paresthesia (Du Four, Mertens et al. 2018).

While a chronic progressive infection of the brain is occasionally reported (Mickiene, Laiškonis et al. 2002, Poponnikova 2008), TBEV is eventually cleared from the brain. Nevertheless, an estimated 40% to 50% of patients experiencing neurological symptoms after TBEV infection develop a post-encephalitic syndrome (Kaiser 2012). The occurring symptoms include memory

and concentration dysfunction, apathy, persistent flaccid paresis and other neuropsychiatric disorders (Haglund and Günther 2003).

TBE severity seems to be dependent on multiple factors. TBEV subtype and dosage of infection seem to play a role (Gritsun, Lashkevich et al. 2003), but also the health status of the infected individual, including age (Logar, Bogovič et al. 2006) and immune status (Kaiser and Holzmann 2000).

The TBEV subtype is not only suspected to be associated with disease severity (Gritsun, Lashkevich et al. 2003, Velay, Paz et al. 2019), but also to the development of uncommon symptoms. Certain strains from the Novosibirsk region are reported to cause hemorrhagic symptoms alongside the typical neurological signs (Ternovoi, Kurzhukov et al. 2003). In Germany, a TBEV-Eu strain was reported that causes no neurological signs and was tentatively linked with mild, mainly gastrointestinal symptoms (Dobler, Bestehorn et al. 2016).

In addition to the infection through a tick-bite, humans can also acquire a TBEV infection through the alimentary route by consuming non-pasteurized dairy products that originated from viremic goats, sheep or cattle. In countries where this form of products is favored, alimentary cases can lead to epidemic outbreaks and pose a threat that the local population is often unaware of (Kohl, Kožuch et al. 1996, Brockmann, Oehme et al. 2018, Bušová, Dorko et al. 2018).

The treatment of TBE is often purely symptomatic and supportive, since there is no specific antiviral drug available. In the past specific immunoglobulins against TBEV were used for treatment and post-exposure prophylaxis, but commercial preparations are no longer available in Europe. This is mostly due to the suspected antibody triggered enhancement of the disease (Bröker and Kollaritsch 2008). Still in Russia and Kazakhstan immunoglobulins are used to this day and reported to prevent or decrease the severity of clinical symptoms (Růžek, Avšič Županc et al. 2019).

Fortunately, reliable vaccines are available, preventing the development of TBE by active immunization (Heinz, Stiasny et al. 2013, Chernokhaeva, Rogova et al. 2018). Therefore, the overall goal in combating TBEV must be to localize endemic regions and warn the local population as well as travelers of the live-threatening disease (Chrdle, Chmelík et al. 2016).

TBE is a disease that is primarily described in humans. Taking into account that the majority of infections in humans progress with no or only mild clinical symptoms (Bogovic, Lotric-Furlan et al. 2010), it is not surprising that TBE is only seldom diagnosed in domesticated animals, although TBEV infects a wide range of mammalian hosts (Klaus, Ziegler et al. 2014). As determined by antibody prevalence studies, dogs are highly susceptible to TBEV infection, but clinical case reports of TBE in dogs are rare. The few that do exist, describe severe neurological symptoms with a fatal outcome in almost all cases. Recovery from TBE does seem to be possible for dogs, but symptoms, like increased aggressiveness and convulsions, can complicate the supportive care measures that are needed to restore the health of the canine patient (Pfeffer and Dobler 2011). Case reports of TBE after a natural infection are even rarer for other animal species. Although horses (Klaus, Horugel et al. 2013), sheep and goats (Klaus, Beer et al. 2012) are highly susceptible to TBEV infection, there is only one case of TBE described for each of this species. In those cases, severe neurological impairment of the affected animal was reported (Waldvogel, Matile et al. 1981, Zindel and Wyler 1983, Böhm, Schade et al. 2017). Furthermore, a moribund mouflon was found in a hunting preserve and post-mortem diagnosed with TBE (Bagó, Bauder et al. 2002). Lastly, a Barbary macaque living in a monkey park in a TBEV risk area fell ill to TBE. It showed a sudden paresis of the hind legs and had to be euthanized after four days due to being comatose (Suss, Gelpi et al. 2007).

## 2.3. Exploring the reservoir hosts of tick-borne encephalitis virus.

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*Review*

# Exploring the Reservoir Hosts of Tick-Borne Encephalitis Virus

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**Abstract:** Tick-borne encephalitis virus (TBEV) is an important arbovirus, which is found across large parts of Eurasia and is considered to be a major health risk for humans. Like any other arbovirus, TBEV relies on complex interactions between vectors, reservoir hosts, and the environment for successful virus circulation. Hard ticks are the vectors for TBEV, transmitting the virus to a variety of animals. The importance of these animals in the lifecycle of TBEV is still up for debate. Large woodland animals seem to have a positive influence on virus circulation by providing a food source for adult ticks; birds are suspected to play a role in virus distribution. Bank voles and yellow-necked mice are often referred to as classical virus reservoirs, but this statement lacks strong evidence supporting their highlighted role. Other small mammals (e.g., insectivores) may also play a crucial role in virus transmission, not to mention the absence of any suspected reservoir host for non-European endemic regions. Theories highlighting the importance of the co-feeding transmission route go as far as naming ticks themselves as the true reservoir for TBEV, and mammalian hosts as a mere bridge for transmission. A deeper insight into the virus reservoir could lead to a better understanding of the development of endemic regions. The spatial distribution of TBEV is constricted to certain areas, forming natural foci that can be restricted to sizes of merely 500 square meters. The limiting factors for their occurrence are largely unknown, but a possible influence of reservoir hosts on the distribution pattern of TBE is discussed. This review aims to give an overview of the multiple factors influencing the TBEV transmission cycle, focusing on the role of virus reservoirs, and highlights the questions that are waiting to be further explored.

**Keywords:** tick-borne encephalitis; ticks; reservoir; transmission; rodent

## 1. Introduction

The term "arbovirus" describes a group of viruses clustered together solely based on their route of transmission. They have managed to adapt to mammalian hosts as well as to arthropod vectors, adapting their replication cycle to two highly different host organisms. In this regard, it seems even more fascinating that arboviruses are found in more than eight virus families, implementing the emergence of this complex system several times in the course of evolution. Today, there are over 500 arboviruses described, including globally recognized threats to human health such as dengue virus, Zika virus, and Japanese encephalitis virus [1,2].

The main factor for the circulation of any arbovirus is the interplay between the arthropod vector and its (reservoir) hosts. To be maintained within a given region, the virus needs to find a system where

there are always sufficient numbers of susceptible hosts for virus amplification and vectors that are able to transmit the virus effectively [3]. Knowing the reservoir of any virus is important to understanding its lifecycle and therefore its distribution. Keeping in mind that this is a complex interplay between many factors, different approaches may sometimes lead to conflicting results. A variety of definitions regarding the term reservoir host are used in the existing literature, with characteristics that often contradict each other [4]. In this review, we present an overview of the current knowledge of the animal hosts involved in the tick-borne encephalitis virus (TBEV) lifecycle and their role in virus maintenance. Without trying to define a classic reservoir host, we aim to highlight the factors contributing to successful virus circulation.

## 2. Tick-Borne Encephalitis: Etiological Agent and Clinical Manifestation

TBEV is one of the main arboviruses in Eurasia, circulating between ticks and vertebrates. It belongs to the family *Flaviviridae*, and within it, to the tick-borne flavivirus group of the genus *Flavivirus* [5]. The genome consists of an approximately 11 kb single-stranded RNA of positive sense, which is packed into an enveloped particle with a diameter of around 50 nm [6]. Based on genome sequence analyses, there are three classic TBEV subtypes described: (I) TBEV-FE (Far East) is found in Asia, mostly in northern China, and in the east of Russia. (II) TBEV-Sib (Siberia) strains are circulating in the rest of Russia, with an outreach to the eastern parts of Europe. (III) TBEV-Eu (Europe) represents the main subtype in mainland Europe [7]. In addition to that, two new subtypes were recently proposed: The Baikalian subtype (TBEV-Bkl), circulating in the region of the Baikal lake [8], and the Himalayan subtype (TBEV-Him), isolated from Himalayan marmots (*Marmota himalayana*) [9]. TBEV evolved in its natural habitat under the constraints of evolution, as part of the specific ecosystem. It adapted to a broad range of species, but remained restricted to natural foci, with strict borders drawn under factors that are still widely unknown to the scientific community [10]. The possible influence of certain weather conditions and adapted host animals is discussed subsequently. TBEV infection leads to the disease tick-borne encephalitis (TBE), also formerly known as Russian spring summer encephalitis (RSSE) in Russia and far eastern Asia, and as Central European encephalitis in the European area [11]. The virus may lead to neurological symptoms varying in severity depending on the subtype. These symptoms may lead to long-lasting sequelae that burden the patient for years after infection, and can also be fatal. Although effective vaccines are available, there are still up to 12,000 cases reported in Europe and Russia each year [12,13]. Disease surveillance in most parts of Asia is not regularly conducted, leaving disease burden estimation to singular outbreak and prevalence studies [14,15]. In addition to human cases, a variety of species are susceptible to TBEV. Rarely, severe clinical symptoms may occur in dogs [16], horses [17], monkeys [18], sheep [19], goats [20], and mouflons [21]. TBEV-specific antibodies have been reported in other animals, such as wild boar, roe deer, or cattle, without clinical disease [22,23].

## 3. TBEV Transmission Cycle: The Tick Vector

For TBEV transmission, the arthropod vectors are primarily hard ticks. In Europe, the most important tick vector is *Ixodes ricinus*, whereas in Russia and Asia it is *Ixodes persulcatus*. In Asia, *Haemaphysalis concinna* also seems to play a major role [24,25]. Other than that, at least 22 tick species have been shown to be able to carry the virus [26–28]. Some may be overlooked because of the lack of human infestation, but still contribute to virus circulation, such as *Dermacentor reticulatus* [29–31]. This highly adaptive tick species is found in large parts of Europe and Asia, and is often the second most common species. In contrast to the only occasional occurrence of human bites, *Dermacentor reticulatus* ticks surpass the number of *Ixodid* tick bites on large domestic and game animals, leading to a potential additional circulation cycle of TBEV [32,33].

The influence of tick population dynamics on TBEV circulation has been reviewed before, highlighting the complex interplay of several factors [34]. In regard to the reservoir function of ticks, two mechanisms play an important role. The virus is maintained in the tick population through

trans-ovarial and trans-stadial transmission, meaning that an infected tick can pass the virus through its eggs to its offspring and that the infected tick carries the virus through all life stages, namely the four development stages: eggs, larvae, nymphs, and adults (Figure 1). Through this inner population circulation, TBEV could possibly transit from an infected egg through all stages to the adult tick and to its eggs again [35]. Although the impact of trans-ovarial transmission is still up for debate [36], the trans-stadial transmission of TBEV is believed to be essential for virus survival in nature, although there are some hints that transmission rates between each stage are not as high as expected [37]. Their long lifespans of up to six years and their ability to survive over winter may also help in retaining TBEV for a long period of time in the same places [38,39]. In addition, TBEV influences the behavior of infected ticks, causing an increase in questing activity [40]. All these factors make the vectors themselves a reservoir for TBEV. However, this alone does not seem to be sufficient for virus maintenance. For successful virus circulation, there needs to be an amplifying host reservoir.

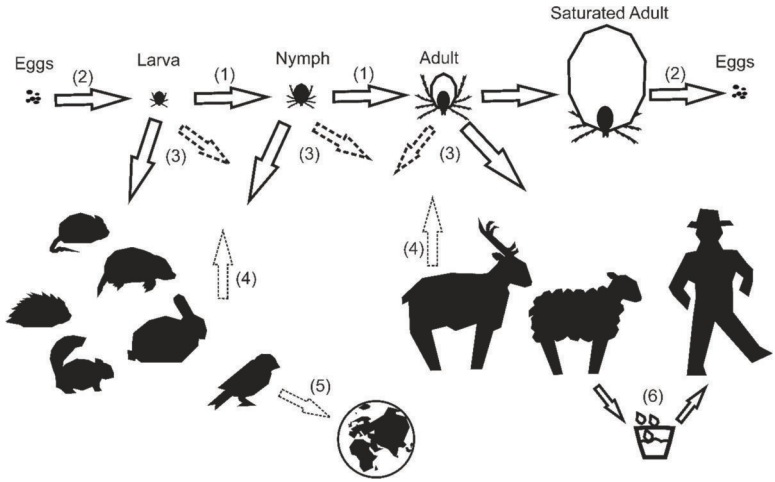


**Figure 1.** Transmission routes of tick-borne encephalitis virus (TBEV): Infected ticks pass the virus to a variety of small and large animals, as well as humans (**3**). Each stage has a preference for certain animal groups ( ), but can be found in a variety of animals ( ). Additionally, humans can become infected by consuming unpasteurized dairy products originating from viremic animals (**6**). Infected birds are suspected to be a vector for virus passage to new endemic foci, although a spatial restriction seems likely (**5**). TBEV is distributed within the tick population mainly through trans-stadial (**1**) transmission, and occasionally through trans-ovarial (**2**) transmission. For successful virus circulation, the virus needs to be spread within the tick population. This is achieved through naïve ticks consuming their blood meal on viremic host animals, as well as through co-feeding (**4**).

## 4. TBEV Transmission Cycle: The Mammalian Reservoir Hosts

For a long time, the consumption of blood from a viremic host by a naïve tick was considered to be the main route of virus dissemination within the tick population. A suitable reservoir host would be an animal that becomes infected with TBEV and keeps the virus circulating in its bloodstream for as long as possible, in titers high enough to infect a feeding tick, without dying from infection, to allow other ticks to feed on it and become infected as well. The effect of co-feeding has also been described, proposing a different method of virus transmission [41]. Through the simultaneous feeding of an infected tick, as well as uninfected ticks in close proximity on the same animal, even when already

immunocompetent against TBEV, successful virus transmission is possible without viremia of the host [42] (Figure 2). This mechanism takes advantage of the relatively long phase of feeding on the host, enabling sufficient virus transmission.

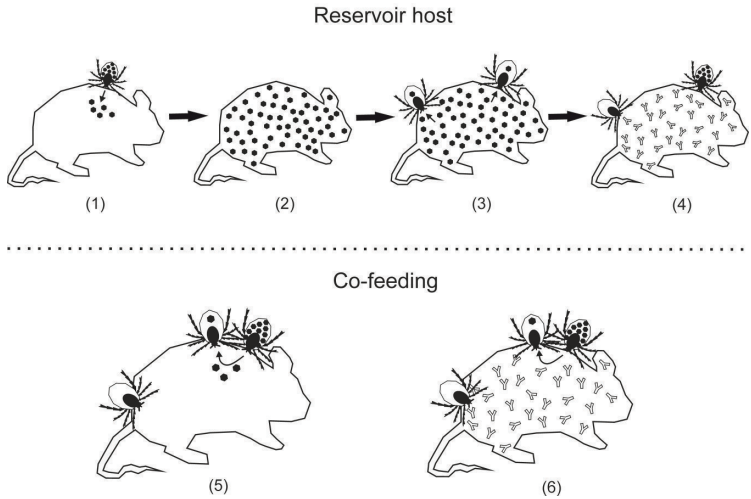


**Figure 2.** TBEV reservoir hosts: Small mammals, especially rodents, are considered to be reservoir hosts for TBEV. Infected ticks transmit the virus (⬢) to the animal host (**1**), leading to viremia (**2**). Naïve ticks acquire TBEV by consuming the blood of a viremic host (**3**). As soon as viremia comes to an end, this route of transmission is blocked by circulating antibodies (⋖) (**4**). Co-feeding enables ticks to pass TBEV among themselves without the need for a viremic host. When naïve ticks feed in close proximity with an infected tick, the animal host acts as a transmission bridge (**5**). This can take place even when the host has antibodies against TBEV (**6**).

Regarding the theory of co-feeding, ticks are considered to be their own reservoir hosts, using the animal to which they are attached as a bridge for transmission. *Ixodes ricinus* and *Ixodes persulcatus* are understood to be the main hosts for TBEV, solely because the larvae and nymphs of these two species tend to have overlapping times of activity, enabling co-feeding between these juvenile stages [43].

Both transmission methods theoretically lead to successful virus amplification and to a spread among the vector population. To which degree the two routes influence the overall virus circulation is still up for debate [44,45].

Human infection can occur through a bite from a TBEV-infected tick when an endemic area is entered. In the period of the year when the tick population reaches its peak, case reports are also at their height, with a delay of three to four weeks. In addition to tick bites, infection through the consumption of unpasteurized dairy products is possible and has now also been reported from Germany [46,47] (Figure 1). In contrast to some mosquito-borne flaviviruses, humans do not play any role in virus transmission, due to low viremia [48] and a lack of sufficient numbers of attached ticks to enable co-feeding. Disease outbreak in humans is theorized to be the result of replication in a not yet adapted host organism [49].

As the main reservoir hosts of TBEV, small mammals such as rodents and insectivores are suspected. In addition to this, an influence of larger game on TBEV prevalence through the influence of mainly adult ticks is discussed (Figure 1). Tick infestation is a major factor in the lifecycle of TBEV, and shows a distinctive pattern for each targeted animal species. There is a general consensus that each

tick stage has a certain range of targeted animals, such as adult ticks mainly targeting large animals, while nymphs and larvae stick to small and medium-sized animals, including birds [50,51].

Larger animals, mainly wild cervids, like roe deer (*Capreolus capreolus*) in Europe, are important hosts for adult ticks [52]. They provide a sufficient blood meal for seeking ticks. The population density correlates with the tick prevalence. A higher number of deer leads to a greater tick population, possible influencing TBEV circulation in a positive way [53].

The role of birds in TBEV circulation is not well understood. Multiple bird species, mainly forest passerines, seem to be able to become infected with TBEV; some may even be able to transmit the virus trans-ovarially to their offspring [54]. Tick infestation on birds seems to be related to the amount of time spent on the ground, mainly because of feeding. The ability of birds to easily cross barriers, such as rivers and highways, enables them to spread attached ticks to new areas that animals living on the forest ground might not reach [55]. If the translocated tick finds a suitable environment with the right climatic and fauna conditions, it could distribute the pathogen it carries [56]. The main role of birds is suspected to be in the dispersion of the virus to new endemic regions, as is theorized for many other tick-related pathogens [57]. As discussed in Klaus et al. [51], dispersion over longer distances seems unlikely for TBEV, since *Ixodes* ticks show a relative short feeding period (5 to 9 days) on their avian host in comparison to the amount of time it takes the bird to cover a certain distance. This leads to early detachment, and therefore to a restriction of the distance covered while being attached to the bird.

A perfect virus amplification reservoir is a host that becomes infected easily, maintains the virus for a long time without causing severe symptoms, and provides a constant stream of naïve individuals [58]. In this sense, for an arbovirus transmitted by ticks, small mammals seem to be a good choice. Through their high reproductive rate and relatively short lifespan, there may always be animals that are naïve to the virus and are able to show viremia after a tick bite. Abundant rodent species in European forests, mainly from the Genera *Myodes* and *Apodemus*, show no reproductive limitation to vegetation season, providing young, naïve individuals even in spring, when tick activity is on the rise [59,60].

Small vertebrate animals live close to the ground and are therefore very easy targets for ticks. In rodents, ticks, particularly nymphs and larvae, aggregate in the area behind the animal's ears, making them an efficient host for transmission through co-feeding. In mainland Europe, the majority of ticks found on trapped rodents originated from only about 20% of captured animals, with two or more nymphs attached to one individual alongside up to 100 larvae. In strong contrast to this, a different picture has been reported from the UK, where only one nymph at most and approximately 10 larvae were found per infested rodent, leading to a nearly 30% increase of the rate of possible co-feeding in European endemic areas in comparison to an area that is naïve to TBEV [61]. In terms of the efficiency of co-feeding, there is a certain dependency on the species it takes place on. The yellow-necked mouse (*Apodemus flavicollis*) seems to be the most adapted species to TBEV and to *Ixodes ricinus* ticks. They show a significantly higher transmission rate than the bank vole (*Myodes glareolus*), which is the second common rodent species in European forests [62]. In addition to this, there are other highly infested small mammals living in tick habitats, such as the two European hedgehog species *Erinaceus roumanicus* [63] and *Erinaceus europaeus* [64], which might provide an equally efficient system.

Studies have provided evidence that TBEV can be passed from experimentally infected voles to their offspring. This vertical transmission allows the virus to circulate within the rodent population without the need for vectors. In the natural reservoir population, this could be a factor that supports long-time virus persistence in a natural endemic focus. However, there are no data available about how passage among only rodent hosts affects the virus and its ability to re-infect arthropod vectors [65]. Experimental infections of suspected reservoir hosts indicate a subclinical infection, with long-lasting virus persistence in the brain. So far, there has been only one study known to us that tried to determine the duration of viremia through PCR analysis of blood samples, indicating a relatively short viremia for the used TBEV-Eu strain, which confirms the results from studies conducted over the last century describing viremia until approximately 4 to 9 days post infection (dpi). In this study, only a single

animal showed viremia for up to 84 dpi when infected with the TBEV-Sib strain. In animals that were inoculated with TBEV-FE, TBEV RNA could be detected in the blood for up to 14 dpi [59,66–68].

## 5. TBEV Prevalence in Wild Small Vertebrate Hosts

In an attempt to locate possible endemic areas, there have been some prevalence studies on wild animals in certain regions. Besides the testing of wild game and farm animals, rodents have been the main focus of surveillance. For this purpose, wild small vertebrate animals were trapped over a certain amount of time and then examined for TBEV contact either through RT-PCR on organ samples or, in most cases, through the detection of antibodies in blood samples [63,69–76] (Table 1).

In Europe, the two rodent species which lead the studies regarding the number of caught individuals are bank voles and yellow-necked mice [63,69–76]. Besides the yellow-necked mice, two other *Apodemus* species were frequently caught in Europe, the wood mouse (*Apodemus sylvaticus*) [69–74,76,77] and the striped field mouse (*Apodemus agrarius*) [69,71,74,75]. Although there were no other *Myodes* species found in these studies, there were some species found from the closely related *Microtus* genus, both *Myodes* and *Microtus* being genera of the subfamily *Arvicolinae*. These species were the common vole (*Microtus arvalis*) [69,72–74,76,77] and the European pine vole (*Microtus agrestis)* [69,72–74,78], as well as the field vole (*Microtus subterraneus*) [74,75] and the tundra vole (*Microtus oeconomus*) [74,76]. All these species, except for the field and tundra voles, for which the number of caught animals was far too low to draw any conclusions for the whole population, seemed to be in constant contact with TBEV, showing antibodies as well as positive RT-PCR results to various degrees throughout Europe [63,69–76]. In Russia, where the area around Novosibirsk has been the main area of investigation so far, a high prevalence of TBEV was also found in the local *Apodemus* and *Myodes* species, namely the striped field mouse (*Apodemus agrarius*) and the northern red-backed vole (*Myodes rutilus*), as well as the grey red-backed vole (*Myodes rufocans*) [79,80]. Furthermore, the common shrew (*Sorex araneus*) and the Northern birch mouse (*Sicista betulina*) were also found in high numbers, with a high percentage of animals found positive for TBEV RNA. The prevalence of TBEV antibodies, as well as the RT-PCR results, found in rodents caught in those areas was considerably higher than in the European studies [79,80].

The grey red-backed vole was also found TBEV-positive in a Japanese surveillance study of the known TBEV endemic region Hokkaido, as well as the large Japanese field mouse (*Apodemus speciosus*), and the small Japanese field mouse (*Apodemus aregenteus*) [81]. In an additional study, mainly conducted in non-endemic regions of Japan, the most caught species, also being the large and small Japanese field mice, showed no signs of contact with TBEV. Additionally, six other caught small mammal species, mainly the Japanese grass vole (*Microtus montebelli*), were found to be negative for TBEV, noting that no grey red-backed vole could be caught in the non-endemic area of Japan [82].

A small study conducted in South Korea found TBEV in striped field mice, offering no information on overall caught rodents but showing the circulation of TBEV in a country where there has been no notified human case of TBE. The sequenced strain clustered with the TBEV-Eu subtype, which is not to be expected in an Asian country [83]. In addition to that, there have been some known isolations of TBE-Eu in Siberia [84]. A possible explanation would be the entry of an infected tick through migratory birds [57], but, as mentioned above, this theory still lacks data and seems unlikely for TBEV. Another possibility is introduction due to the massive worldwide movement of goods. The importance of these anthropogenic factors in the distribution of TBEV has been shown in a phylogenetic study by Kovalev et al. [85], linking the spread of TBEV-Sib throughout Russia to the construction of the Trans-Siberian Way [3].

In Finland, a study was carried out in two different trapping sites, comparing a TBEV-Sib endemic region with another one endemic for the European subtype. The location, known for the circulation of TBEV-Eu, found field voles as a dominating TBEV-infected species. No bank voles or yellow-necked mice were caught at this site. The TBEV-Sib locus found bank voles, the main species of the other European studies, with TBEV antibodies, as well as the virus in organ samples [78].

Closely related species seem to be able to take over the role as the main reservoir host in the absence of the original host, with field voles taking over for bank voles, and bank voles also being able to circulate TBEV-Sib virus strains [78]. However, due to the small sample size, this might just be the result of local infection pressure, rather than an actual adaption to the respective species. Bank voles were also shown to be susceptible to all three subtypes by experimental infection [66]. Similar to these findings in rodents, tick species seem to be equally susceptible to virus strains of variable subtypes. In studies conducted in Finland, in field-collected *Ixodes persulcatus*, the European subtype was found, and the Siberian subtype was also detected in *Ixodes ricinus* [86,87]. There is a consensus that *Ixodes ricinus* and *Ixodes persulcatus* are the main driving forces for the relatively strict distribution of virus subtypes [27]. Since these studied areas are on the border between the two subtypes, they offer a good place to investigate virus evolution, as well as the interface of the different hosts. Finding one tick positive for an unsuspected subtype may be seen as proof of adaption in different tick species. In addition to this, there is a division inside the *Ixodes persulcatus* population, with two races showing significant variation in morphometric parameters, aligning with the geographic distribution of TBE-Sib and TBE-FE [88].

Although there is no striking connection between host animals and endemic regions, a closer examination of supposedly homogenous mammalian populations could offer an explanation. While there is a concordant geographical distribution of genetic lineages of various animal species around the majority of the world, in Europe, such a pattern cannot be found. In contrast, studies based on mitochondrial DNA analysis reveal distinct distribution patterns of lineages even between mammalian species of the same genus, leading to a high ecological plasticity of many species across Europe [89]. Difference between different lineages, in particular relating to the immune system, might make a species much more diverse than predicted [90].

Next-generation sequencing could be the key to discovering differences within lineages of animal species that might be responsible for different reactions to virus infection, and, as a consequence, potentially influence the development of TBEV endemic areas [91]. A similar situation has already been shown for the distribution of *Puumala orthohantavirus*, since the spatial distribution of this virus is connected to different linages of its reservoir host [92]. Considering bank voles as a potentially important reservoir for TBEV, the relatively closely studied lineage distribution has shown an alliance with TBEV risk areas [93,94].

**Table 1.** Small mammalian animals caught in TBEV studies worldwide. Studies focusing on antibody prevalence are shaded in grey; the remaining studies were conducted by screening for viral RNA. CHE—Switzerland; CZE—Czech Republic; DEU—Germany; FIN—Finland; HUN—Hungary; JPN—Japan; KOR—South Korea; RUS—Russia; SVK—Slovakia; SVN—Slovenia.

| **Genus** | | **Apodemus** | | | **Myodes** | | | | **Microtus** | | **Sorex** | | **Sicista** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Species*** | | ***A. flavicollis*** | ***A. sylvaticus*** | ***A. agrarius*** | ***My. glareolus*** | | ***M. arvalis*** | ***M. agrestis*** | ***M. subterraneus*** | ***M. oeconomus*** | ***S. araneus*** | **S. sp.** | |
| | | **pos./total** | **pos./total** | **pos./total** | **pos./total** | | **pos./total** | **pos./total** | **pos./total** | **pos./total** | **pos./total** | **pos./total** | |
| Country | Publication | | | | | | | | | | | | |
| CZE | [73] | 2/144 | 0/17 | | 2/92 | | 0/8 | 0/3 | | | | | |
| | [72] | 0/77 | 0/34 | | 1/41 | | 0/2 | 0/1 | | | 0/1 | | |
| SVN | [71] | 33/820 | 7/66 | 4/160 | 39/272 | | | | | | | | |
| SVK | [63] | 18/290 | | | 2/14 | | | | | | | 2/12 | |
| | [76] | 130/717 | 36/408 | | 233/1538 | | 14/161 | | | 0/2 | 4/29 | | |
| HUN | [74] | 4/100 | 0/11 | 4/55 | 6/150 | | 3/48 | 0/2 | 0/31 | 0/8 | | | |
| | [75] | 12/327 | | 8/174 | 8/39 | | | | 0/1 | | | | |
| DEU | [69] | 10/123 | 2/7 | 3/24 | 21/163 | | 2/21 | 7/101 | | | | | |
| | [77] | 14/103 | 1/19 | | 14/91 | | 1/2 | | | | | | |
| CHE | [70] | 1/77 | 3/104 | | 8/152 | | | | | | | | |
| FIN | [78] | | | | 12/80 | | | 17/95 | | | 0/23 | | |
| ***Species*** | | ***A. agrarius*** | | | ***My. rutilus*** | ***My. rufocans*** | | | | | ***S. araneus*** | | ***Si. betulina*** |
| | | pos./total | | | pos./total | pos./total | | | | | pos./total | | pos./total |
| Country | | | | | | | | | | | | | |
| KOR | [83] | 5/24 | | | | | | | | | | | |
| RUS | [79] [1] | 12/34 (16/34) | | | 25/32 (37/45) | 18/39 | | | | | 22/30 | | 14/18 |
| | | pos.% | | | pos.% | | | | | | pos.% | | |
| RUS | [80] | 43.3 ± 9 | | | 80.0 ± 9.2 | | | | | | 69.2 ± 12.8 | | |
| | | 40.6 ± 8.7 | | | 61.9 ± 10.8 | | | | | | 83.3 ± 6.8 | | |
| ***Species*** | | ***A. speciosus*** | ***A. aregenteus*** | | ***My. smithii*** | ***My. rufocans*** | ***M. montebelli*** | ***M. minutus*** | | | ***S. unguiculatus*** | ***S. sp.*** | |
| | | pos./total. | pos./total. | | pos./total. | pos./total. | pos./total. | pos./total. | | | pos./total. | pos./total. | |
| Country | | | | | | | | | | | | | |
| JPN | [81] | 4/24 | 1/37 | | | 14/95 | | | | | 0/6 | 0/2 | |
| | [82] | 2/455 | 0/36 | | 0/24 | | 0/47 | 0/1 | | | | 0/5 | |

[1] RT-PCR was performed on brain as well as blood cell samples (shown in parentheses) from the same animals.

## 6. Distribution

Over the last few decades, the prevalence of TBEV has been increasing and more endemic regions have been described [95]. This is, on one hand, due to improved surveillance and increased awareness of the possible TBEV infection of most patients suffering from encephalitis. On the other hand, higher temperatures are leading to prolonged tick activity and an increased geographical distribution of ticks, in particular, in northern European countries [96]. Combined with a change in leisure activity, which leads to more frequent visits to tick habitats, this increases the possibility of contact between humans and infected ticks [95].

Multiple factors play a role in the development of a TBEV endemic region. Certain botanical, zoological, climactic, and geo-ecological conditions need to be fulfilled to create a suitable environment for virus circulation [97]. A temperature level of more than 7 °C and a relative humidity of over 80% for most of the time create a suitable tick environment. These conditions are found mainly in forests and grassland areas with sufficient rainfall [96,98]. With regard to TBEV, there are some theories about certain weather conditions promoting the virus circulation [99–101]. For example, a rapid fall in ground-level temperatures in early autumn seems to prepone the activity of larvae, adjusting it to the main activity period of nymphs. The resulting enhanced synchronicity of larvae and nymph activity allows a prolonged period of co-feeding between ticks of both stages, and increases the virus transmission rate inside the local tick population [102]. In addition to this, the mere presence of several larvae on the same animal seems to play a major role. Mass co-feeding of larvae in spring as well as in autumn also seems to contribute to virus distribution between ticks to a considerable extent [36].

While the eastern subtypes seem to show quite a homologous distribution alongside the tick population, the European subtype shows a different pattern [102]. Showing lower prevalence in ticks and caught wild rodents, the circulation of TBEV-Eu seems to be restricted by certain factors. Even though ticks and small mammals can be found all around the European continent, TBEV is not endemic in large parts, namely in the west.

Inside an endemic region, TBEV exhibits a specific distribution. In contrast to other tick-borne pathogens like *Borrelia burgdorferi* s.l., TBEV-Eu is not found evenly among the tick population, but is clustered to certain areas from about a few square meters to several square kilometers in size [103,104]. These so-called "natural foci" are believed to be their own autonomous ecosystems, although not showing any striking ecological differences to the surrounding area. There are indications for a center of virus maintenance, in which a constant high infection rate is found in ticks, and which is surrounded by an area where pathogen circulation is significantly lower [105]. Based on studies of two endemic regions in Bavaria, the actual area of a circulating virus strain in this certain area was estimated to be only around 2.500 square meters, with the virus circulating between ticks and small rodents. Out of these so-called "microfoci," infected ticks may be brought out of the reservoir through medium- and large-sized wild animals. This may lead to transmission in an area of about one kilometer in diameter around the microfocus, described as the "macrofocus" [106]. Existing foci seem to be able to develop in different ways. A study comparing recent data with results from 40 years prior in Thuringia showed that singular foci evolved differently despite being in the same area. Areas with foci of low TBEV incidence showed more human cases of TBEV, while one high-risk focus disappeared completely [107].

## 7. Situation of other Tick-Borne Flaviviruses

When studying the TBEV reservoir, it may also help to take a closer look at its close relatives that induce similar clinical symptoms. Powassan virus (POWV) is a flavivirus from the tick-borne encephalitis serogroup that is mainly found in eastern Russia and North America, including parts of Alaska [108]. Vector ticks are mainly the local *Ixodid* species, like *Ixodes scapularis* and *Ixodes cookei* in America. In Siberia, *Haemaphysalis longicornis* is known to transmit POWV [109]. A serological survey was conducted to get an overview of the POWV prevalence in free-ranging small vertebrate animals. Although there was no further specification about the detected flavivirus, the study gave the first hint of a correlation between small animals and POWV outbreaks. In Siberia and central Alaska, the only

species found to be positive for antibodies against the flavivirus serogroup, and by far the most caught species, was the northern red-backed vole (*Myodes rutilus*), which is the same species that dominated Russian survey studies for TBEV [110].

In the south of Alaska, the role of the northern red-backed vole is taken over by the southern red-backed vole (*Myodes gapperi*). In southwestern USA, no *Myodes* species was caught. The most frequently trapped animals were from different species of the genus *Peromyscus*, a genus from the same family as the genus *Myodes*. Some of them were seropositive for some kind of flavivirus that could not be further characterized [110]. In a study conducted in the eastern part of the USA, a *Peromyscus* species, the white-footed mouse (*Peromyscus leucopus*), also made up the largest portion of caught animals. Even though POWV was successfully isolated from ticks from the same area and antibodies against POWV were detected in slightly larger mammals like woodchucks (*Marmota monax*), no antibodies could be found in the white-footed mouse [111].

While POWV shows a similar reservoir situation to TBEV, the tick-borne flavivirus that dominates in Britain, namely the louping ill virus (LIV), has adapted in a different way to the local circumstances. LIV is another tick-borne flavivirus that seems to have only relatively recently diverged from the TBEV complex [112]. The vector tick is *Ixodes ricinus*, the same tick species that transmits TBEV in Europe. However, LIV seems to have adapted to the different environmental conditions of Britain, leaving the woodlands of Europe for the locally more frequent upland moors, switching to sheep (*Ovis aries*) and red grouse (*Lagopus lagopus scoticus*) as the main hosts and resulting in a complete abandonment of small rodents as an important reservoir [113]. LIV also causes actual disease in sheep, switching the main concern to economic losses in agriculture, rather than human infection [114].

## 8. Discussion

TBEV is a tick-borne virus circulating among mainly tick vectors and a variety of vertebrate hosts, and, as in any other biological system, many factors contribute to its lifecycle. Hard ticks play a major role in the distribution of the three virus subtypes across Europe and the northern parts of Asia. Although there is a classic view of small mammals being the major reservoir hosts for virus circulation, there are other important factors that should not be overlooked. Ticks themselves represent a reservoir, circulating the virus within their population mainly through trans-stadial transmission for long time periods. *Myodes glareolus* and *Apodemus flavicollis* make up the majority of mammals caught in European TBEV surveillance studies, and are consistently found positive for antibodies against TBEV, as well as for viral RNA. In Japan, this role is taken up by the local species of the same genera, namely *Apodemus speciosus* and *Myodes rufocans;* in Russia it is *Apodemus agrarius* and *Myodes rutilus*, alongside a high percentage of *Sorex araneus*. Nevertheless, there is a lack of studies on the true potential of these rodents as classic virus reservoirs for TBEV. Most studies, dating back to the middle of the 20th century and mainly written in Russian, go widely unnoticed by the recent scientific community [115]. Since the only recent study concerning viremia of natural rodent hosts hints at a relatively short viremia for TBEV-Eu [66], high prevalence findings within the rodent population of Europe might be a consequence of contact to TBEV-positive ticks. There are only few scientific data about the virus titer of viremia in a potential reservoir host needed for efficient virus transmission [116]. The high titers of TBEV-RNA found in organ samples for a relatively long time after experimental infection could enable active virus transmission through rodents. As shown for other pathogens, tick saliva is able to act as a chemotactic agent, reactivating pathogens and enabling attached ticks to still become infected [42,117].

There is a need for additional experimental infection studies, as well as studies in natural environments, as standardized laboratory conditions might affect the results [118]. Without further proof for the role of ticks as important reservoir hosts, further studies should also focus on a broader range of mammalian hosts. There have been almost no survey studies on other animal species living in tick habitats that might not be trapped in standard devices and do not belong to typical game animals. A study from Kožuch et al. indicates that hedgehogs and dormice (*Glis glis*) seem to be able to carry

viruses through hibernation, which might play a role in virus maintenance, but clearly needs further analysis [119]. In regard to co-feeding, there is a lack of further studies after the initial studies made by Labuda [41,62], leading to an acceptance of the mechanism as a side transmission route without exploring its true significance for the overall TBE lifecycle. Furthermore, there are no data available concerning the same effect with the eastern branches of ticks, viruses, and small mammalian animals. Since tick stages seem to meet on a huge variety of animals, co-feeding might be possible on other hosts as well.

The TBEV lifecycle still offers many unanswered questions ready to be explored, especially if we want to understand its influence on the typical focal distribution of endemic regions. A lot of influence seems to stem from climatic conditions on ticks themselves, as well as on their food sources. Fluctuations of rodent, deer, and tick populations seem to play an unclear role in productive virus transmission. Overall, there is a need for further investigation into the often highlighted role of particular rodent species as a virus reservoir. More in-depth studies of known natural foci and experimental studies on suspected rodent reservoir hosts may provide a better understanding of the complex TBEV lifecycle.

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## 2.4.*In vivo* studies in natural hosts

In early TBEV research, a variety of animal species were experimentally infected with TBEV (Gresikova 1958, van Tongeren and Timmers 1961, Grešíková, Weidnerová et al. 1972, Votiakov, Protas et al. 1975, Pogodina, Levina et al. 1981), with a focus on presumed reservoir hosts like bank voles and yellow-necked mice. A viremia of three to nine days post infection (dpi) was shown as well as virus in the brain of some bank voles even after 28 days (Ernek, Kožuch et al. 1963, Heigl and Zeipel 1966, Zeipel and Heigl 1966, Chunikhin and Kurenkov 1979). The characterization of different TBEV strains showed different levels of viremia (Chunikhin, Kurenkov et al. 1981, Kozuch, Chunikhin et al. 1981). Studies on hibernating animals, showed that hedgehogs (*Erinaceus roumanicus*) and dormice (*Glis glis*) develop a viremia of eight and up to 36 dpi, respectively, after a hibernation period of one month (Kožuch, Grešíková et al. 1967). Also the European mole (*Talpa europaea*) was infected and showed a viremia until 10 dpi (Kožuch, Grulich et al. 1965).

The discovery of co-feeding (Labuda, D Jones et al. 1993) and the fact, that this transmission process can take place even on natural hosts that are already immune to TBEV changed the view on the role of potential reservoir hosts (Labuda, Kozuch et al. 1997, Randolph 2011). Nevertheless, it is believed that multiple transmission ways are needed to ensure the persistence of TBEV. A vertical transmission of TBEV was shown not only in laboratory mice (Gerlinskaya, Bakhvalova et al. 1997) but also in experimentally infected red voles (*Myodes rutilus*) (Bakhvalova, Potapova et al. 2009).

A small study performed in the common vole *(Microtus arvalis*) showed a viremia of 50 dpi, by detection of viral RNA in blood samples. In addition, virus was successfully cultivated from brain samples that were taken 100 dpi (Achazi, Růžek et al. 2011). The most recent study of TBEV in a natural host was performed in 2013. The three classical TBEV subtypes were *in vivo* characterized in the bank vole. Similar to earlier studies, a persistent infection of the brain was shown. Viremia was determined by detection of viral RNA in serum samples. All three subtypes led to viremia for 14 days in some animals, a single animal was tested positive 25 dpi and one 84 dpi. The two last-mentioned animals were infected with the Far Eastern and the Siberian TBEV subtype respectively (Tonteri, Kipar et al. 2013).

# 3. Objectives

**Interaction between TBEV and its natural animal host**

The goal of the presented study was to characterize the interaction between TBEV and its natural host. Although TBEV infection was shown in various wild animal species, small ground-living mammals are suspected to take on an important role in the TBEV transmission cycle, since they live in close proximity to the vector tick (Cayol, Jääskeläinen et al. 2018). As a representative the bank vole was chosen. The bank vole is one of the most prevalent species in European forests. According to that, the TBEV strains that were used in this study all belonged to the European TBEV subtype. A variety of different strains differing in the place and organism they originated from were comparatively tested in a newly established bank vole infection model. In addition, non-inoculated bank voles were kept as in-contact controls to see if horizontal transmission of TBEV can take place within the bank vole population.

**Influence of regional bank vole lineages on TBEV transmission**

Bank voles are found in different lineages in Europe due to the post-glacial repopulation of the mainland, after the hiding out of several colonies in different refugia (Wójcik, Kawałko et al. 2010). As already shown for Puumala Orthohantavirus (Drewes, Ali et al. 2017), these local linages can play a role in the transmission cycle of certain viruses. Similar differences in the virus dynamics of TBEV in these lineages might be possible. Therefore, selected TBEV strains were tested in two different bank vole lineages to study the interaction between local occurring TBEV strains and the respective bank vole lineage.

**Evaluation of TBEV detection methods for surveillance in wild-caught rodents**

In Europe, even in endemic regions, TBEV can be detected in only approximately one in 1000 ticks (Steffen 2016). Therefore, surveillance programs rely on the determination of antibody prevalence and TBEV RNA detection in wild-caught animals. Different sample matrixes generated from the *in vivo* infection studies were evaluated in the presented study in order to identify the most suitable sample materials from voles for TBEV antibody and viral RNA detection.

# 4. Results

## 4.1. In vivo characterization of tick-borne encephalitis virus in bank voles (*Myodes glareolus*).

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# In Vivo Characterization of Tick-Borne Encephalitis Virus in Bank Voles (*Myodes glareolus*)

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**Abstract:** Tick-borne encephalitis is the most important tick-transmitted zoonotic virus infection in Eurasia, causing severe neurological symptoms in humans. The causative agent, the tick-borne encephalitis virus (TBEV), circulates between ticks and a variety of mammalian hosts. To study the interaction between TBEV and one of its suspected reservoir hosts, bank voles of the Western evolutionary lineage were inoculated subcutaneously with either one of eight TBEV strains or the related attenuated Langat virus, and were euthanized after 28 days. In addition, a subset of four strains was characterized in bank voles of the Carpathian linage. Six bank voles were inoculated per strain, and were housed together in groups of three with one uninfected in-contact animal each. Generally, most bank voles did not show any clinical signs over the course of infection. However, one infected bank vole died and three had to be euthanized prematurely, all of which had been inoculated with the identical TBEV strain (Battaune 17-H9, isolated in 2017 in Germany from a bank vole). All inoculated animals seroconverted, while none of the in-contact animals did. Viral RNA was detected via real-time RT-PCR in the whole blood samples of 31 out of 74 inoculated and surviving bank voles. The corresponding serum sample remained PCR-negative in nearly all cases (29/31). In addition, brain and/or spine samples tested positive in 11 cases, mostly correlating with a positive whole blood sample. Our findings suggest a good adaption of TBEV to bank voles, combining in most cases a low virulence phenotype with detectable virus replication and hinting at a reservoir host function of bank voles for TBEV.

**Keywords:** tick-borne encephalitis virus; bank vole; experimental infection; virus detection; reservoir host

## 1. Introduction

Tick-borne encephalitis (TBE) is a severe neurological disease that can lead to long-lasting sequelae, burdening the affected patient for years [1]. Even though effective vaccination is possible, there are still 2000 to 4000 cases reported yearly in the European Union alone, where TBE has been a notifiable disease since 2012 [2]. Worldwide, there are more than 10,000 cases reported each year; the highest percentage

of cases diagnosed in Russia [3]. Since TBE surveillance in the northern parts of Asia is not yet regularly conducted except for in Russia, the actual number of cases may be even higher [4,5]. Overall, case numbers tend to fluctuate over time [6,7], since transmission rates to humans are dependent on multiple factors [8–10]. The causative agent, the tick-borne encephalitis virus (TBEV), is a member of the family Flaviviridae, in which it belongs to the tick-transmitted complex alongside with louping-ill virus (LIV), Langat virus (LGTV), Kyasanur Forest disease virus (KFDV), and Powassan virus (POWV), and a number of other viruses [11]. TBEV is divided into at least five subtypes: the European subtype (TBEV-Eu), the Siberian subtype (TBEV-Sib), the far-eastern subtype (TBEV-Fe), and the recently identified Himalayan and Baikalian subtypes [12–14]. Among factors such as the infectious dose, age, genotype, and health status of the patient [15], the subtype can influence the severity of disease in humans [6,16,17]. Immune response to TBEV infection may also play a role in disease severity and has been reviewed by Ruzek et al. [15]. Hard-bodied ticks are the central point of the transmission cycle of TBEV [6,18,19]. They spread the virus among a variety of animal species [20–24] and represent a virus reservoir, as they are able to retain the virus during their different life stages through trans-stadial and trans-ovarial transmission [25]. Nonetheless, an additional source of infection for naïve ticks is needed to spread the virus in the tick population and assure sufficient circulation in endemic regions [26]. This source of infection is often presumed to be a vertebrate reservoir. According to the WHO, a reservoir host is a mammalian host that ideally becomes infected without showing signs of disease and remains viremic for a long time, with titers high enough to infect a naïve vector [27]. However, there is no unified definition of the term reservoir host [28] and, therefore, there are no clear criteria a reservoir host has to fulfil [29]. In this paper, the term "reservoir host" is therefore used merely as a term to define the possibility of a host to become a relevant source of infection for an arthropod vector through the development of long-lasting viremia. The suspected vertebrate reservoir hosts for TBEV are small mammalians living on the ground of the deciduous and mixed forest ecosystems where ticks are found in abundance [30]. Alongside a process called co-feeding, where infected ticks pass the virus directly to naïve ticks through a shared feeding pool while being attached to the same animal in close proximity [31], the classical route of infection is via consumption of a blood meal from a viremic animal [32]. However, the importance of this direct transmission of TBEV from a viremic animal to a naïve tick has been questioned [33], mostly based on the fact that there are hardly any studies available on the interaction between TBEV and its putative natural hosts. Existing studies describe a viremia of three to nine days and a possible persistent infection of the brain of various small mammalian species [34–38]. A more recent study described a potentially longer viremia, especially after an infection with a TBEV-Fe strain in bank voles [39].

The present study set its focus on the situation in Europe, where *Ixodes ricinus* ticks are the main vectors and bank voles (*Myodes glareolus*) are suspected to be one of the main vertebrate reservoir hosts [40]. Bank voles are among the most frequently trapped small mammals in various European TBE monitoring studies. They are used as sentinels for TBEV circulation since both antibodies and viral RNA in considerable amounts have been found in organ samples of caught animals from known endemic regions [41]. The bank vole population is divided into different evolutionary lineages based on mitochondrial DNA (mtDNA) sequencing. These lineages originated due to the post-glacial re-colonization of Europe from bank vole colonies that survived the glaciation in different refugia. The Western lineage is found in the western parts of Europe and is separated from the Eastern lineage by the Carpathian lineage which occurs in Poland, the Slovak Republic, and Romania. In addition, Spanish, Italian, and Balkan lineages have been described [42].

Here, a variety of TBEV-Eu strains that were isolated from either humans, ticks, or bank voles were selected and inoculated into bank voles of the Western evolutionary linage [42]. In addition, LGTV was used, which is a lowly pathogenic virus that is similar to TBEV in its transmission cycle but not endemic in Europe [43]. Furthermore, LTGV shows antibody cross-reactivity with TBEV and was considered a vaccine candidate in early TBEV research [44]. To address the potential influence of different linages on the interaction between TBEV and the natural rodent host, as is known for,

for example, Puumala orthohantavirus [45], four of these strains were also tested in bank voles of the Carpathian linage.

The samples that were generated during this experimental infection study were further used to validate available test systems for the bank vole and to evaluate different sample matrices for their usage to detect certain parameters.

## 2. Materials and Methods

### *2.1. TBEV-Eu Strains*

Eight TBEV-Eu strains were selected (Table 1). Seven strains were obtained from the collection of the Department of Microbiology of the German Armed Forces, Munich, Germany. The eighth strain (IZ58) and the LGTV were obtained from the virus collection of the Friedrich-Loeffler-Institut, Greifswald—Insel Riems, Germany. The selected strains were propagated on A549 cells (L 1035, Collection of Cell Lines in Veterinary Medicine (CCLV), Friedrich-Loeffler-Institut, Greifswald—Insel Riems, Germany) for one passage.

**Table 1.** Virus strains used in the present study, including information on the initial isolation (year, place, species).

| Strain | First Isolation | | | | Passage on Cell Culture | Accession Number (NCBI GenBank) | Reference |
|---|---|---|---|---|---|---|---|
| | Year | Country | Location | Species | | | |
| BaWa 15/943 | 2015 | GER | Haselmühl | tick | 1× | - | - |
| HB 171/11 | 2011 | GER | Heselbach | tick | 2× | KX268728 | Dobler et al., 2016 [46] |
| IZ58 | 1965 | GER | Schorfheide | tick | 3× | - | Apitzsch et al., 1968 [47] |
| Neudörfl | 1970 | AUT | Neudörfl | tick | n.a. | U27495 | Mandl et al., 1988 [48] |
| Battaune 17-H9 | 2017 | GER | Leipzig | bank vole | 1× | - | - |
| CGl 223 | 1990 | SVK | Záhorská Ves | bank vole | 1× | KC835597 | Kozuch et al., 1995 [49] |
| HM 4-2 | 2015 | GER | Haselmühl | bank vole | 2× | - | - |
| Scharl | 1956 | AUT | Lower Austria | human | n.a. | - | Ecker et al., 1999 [12] |
| Langat virus | 1956 | MYS | Kuala Lumpur | tick | 3× | - | Smith 1956 [43] |

The number of passages on cell culture between first cultivation and the usage in the animal experiment is indicated. Passages of two isolates were not available (n.a.). The accession numbers refer to the full-length sequence of the respective strain. Austria: AUT, Germany: GER, Malaysia: MYS, SVK: Slovak Republic.

### *2.2. Animals and Experimental Design*

All seven TBEV-Eu strains as well as LGTV were inoculated into bank voles of the Western linage. Four out of these TBEV-Eu strains, namely Scharl, Battaune 17-H9, GCl 223, and IZ58, were simultaneously characterized in bank voles of the Carpathian linage.

Animal housing and all handling took place under BSL 3** conditions. Altogether, 114 outbred bank voles (*Myodes glareolus*) obtained from the in-house breeding colonies of the Friedrich-Loeffler-Institut were used. The breeding colony of the Western evolutionary lineage originated from bank voles that were provided by the Federal Environmental Agency in Berlin, Germany, and the breeding colony of the Carpathian evolutionary lineage originated from bank voles that were provided by Jagiellonian University Krakow, Poland. Serological assays are performed on a regular basis to ensure the specific pathogen-free status of both breeding colonies [50]. PCR amplification and sequencing of the partial *cytochrome b* gene was performed following a standard protocol [51]. The generated nucleotide sequences were then used in a phylogenetic analysis to confirm their affiliation to the respective evolutionary lineage [50]. Seventy-eight bank voles belonged to the Western lineage and 36 to the Carpathian lineage. The voles were kept in single-ventilated type III mouse cages under the following conditions: 22 °C; 12/12 h light cycle, approximately 60% humidity, water and rodent pellets ad libitum. To assure smooth social interaction between the voles, only female voles were selected. Admittedly, three animals turned out to be males at dissection. The animals were housed in pairs of four, ranging in age between 5 and 32 weeks at the day of infection. Three voles from each cage were inoculated subcutaneously with 100 µL virus dilution per animal, containing $10^5$ tissue

culture infectious dose 50% ($TCID_{50}$). The remaining animal acted as an in-contact animal to detect possible transmission from the infected voles. For each TBEV-Eu strain, a total of six voles were inoculated, meaning that two cage groups of three voles with one contact animal each were used per strain. Ten voles acted as environmental controls; six out of them belonged to the Western lineage and four to the Carpathian lineage. All voles were examined daily based on a clinical score system (up to three points were awarded for each changes in behavior, neurological symptoms, and loss of body weight). Weight loss of more than 20% of the original weight, paralysis of the limbs, a clinical score of seven or other clinical signs suggesting suffering were predefined as endpoint criteria. Twenty-eight days post infection (dpi), autopsy of all remaining bank voles was performed. In addition to the collection of whole blood and serum samples, 11 organs (brain, spinal cord, lung, heart, small and large intestine, liver, spleen, kidney, bladder, and uterus/testicle) were sampled. Whenever possible, samples of feces and urine were taken as well. Lastly, a lavage of the chest cavity was performed with 1 mL phosphate-buffered saline buffer (PBS). All samples were stored at −80 °C until analyzed.

The experimental design was evaluated and approved by the relevant state ethics committee (State Office for Agriculture, Food Safety and Fishery in Mecklenburg-Western Pomerania, permission number 7221.3-1.1-029/18, 28 May 2018).

### *2.3. RNA Extraction and RT-PCR*

The collected organs and the feces samples were mixed with 1 mL modified Eagle's medium (MEM) and homogenized using a TissueLyzer (Qiagen, Hilden, Germany). After centrifugation, 100 µL of the supernatant was used for RNA extraction. Urine samples were also collected in 1 mL MEM, of which 100 µL was used for extraction. Lavages were used directly (volume 100 µL). For the extraction of RNA from EDTA blood and serum, 15 µL of the sample was used. RNA extraction was performed using the King Fisher 96 Flex purification system (Thermo Scientific, Braunschweig, Germany) in combination with the NucleoMag® Vet Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The extracts were subsequently tested for TBEV using a previously described and validated real-time RT-PCR, targeting a fragment of the 3'-untranslated region (3'UTR) of the TBEV genome [52]. The TBEV test was carried out as described; however, to control for efficient RNA extraction and amplification and thereby avoid false negative results, an internal control based on the beta-actin gene was included [53] instead of the previously described heterologous control [52].

### *2.4. Comparison of Real-Time RT-PCR to Cell-Culture Infectivity*

TBEV-Eu cell culture passages that were used in the animal experiment (Table 1) were used to correlate cell-culture infectivity to real-time RT-PCR detection of viral genome. To determine the cell-culture infectivity, the viral suspension of each isolate was diluted in serum-free MEM in a 10-fold series until a dilution of $10^{-8}$ was reached. A459 cells suspended in MEM supplemented with 5% bovine viral diarrhea virus (BVDV)-free fetal calf serum were then added to each dilution. The described titration was performed in eight replicates and $TCID_{50}$/mL was determined through detection of cytopathic effect (cpe).

For the detection of viral genome, the viral suspensions were diluted using serum-free MEM in a 10-fold dilution series until a $10^{-12}$ dilution was reached. Viral RNA was extracted and real-time PCR was performed as described above, using 100 µL of each dilution for the initial extraction. The mathematical relationship between real-time RT-PCR and the logarithmic $TCID_{50}$/mL values of the same dilution was then modeled using simple linear regression [54]. The SigmaPlot program (Sytat Software GmbH) was used to create a graph with a single linear regression line for all TBEV-Eu strains. RT-PCR results were then used to estimate cell-culture infectivity.

### *2.5. Virus Isolation*

Reisolation of viruses in cell culture was attempted on human lung carcinoma cells (A549, L 1035 CCLV, Insel Riems, Germany), cultivated in MEM supplemented with 10% BVDV-free fetal calf serum

for three passages. Successful cultivation was detected through cpe on the cells and confirmed through RT-PCR.

### *2.6. Antibody Detection*

A microneutralization assay was performed according to Holzmann et al. [55], with minor modifications. Each serum sample was tested in duplicate. A set of known control sera was tested in parallel. The serum samples were first diluted in a 1:20 ratio and then titrated in 2-fold dilutions. LGTV was then added with approximately 100 $TCID_{50}$/well, which was confirmed by performing back-titrations. A549 cells were added to the virus–serum mixture and incubated at 37 °C for seven days. Titers were evaluated via appearance of cpe and are expressed as the dilutions that caused 50% neutralization ($ND_{50}$). Besides the collected sera, the chest cavity lavages were also tested by the microneutralization assay, following the same protocol but starting at a dilution of 1:5.

## 3. Results

### *3.1. Clinical Manifestation*

None of the bank voles showed any neurological symptoms over the course of infection. A single animal of the Western lineage (inoculation with strain Battaune 17-H9) displayed signs of distress and died three days after infection, before the clinical examination score fulfilled the predefined humane endpoint criteria.

Two voles of the Western lineage and one of the Carpathian lineage, inoculated with the same TBEV-Eu strain (Battaune 17-H9), had to be euthanized at 5, 6, or 12 dpi due to weight loss of more than 20% of the animal's original weight. For the same reason, one contact animal, which belonged to the Western lineage, had to be euthanized 6 days after infection as well as one environmental control animal, which belonged to the Carpathian lineage, at 19 dpi; none of these animals displayed any signs of distress except for weight loss.

### *3.2. Virus RNA Detection*

In general, EDTA blood represented the sample material that most frequently tested positive by real-time PCR in both vole lineages. At the end of the study (28 dpi), viral RNA was detected in whole blood samples of 31 animals out of the 74 surviving inoculated voles. The respective viremic animals had been inoculated with either the TBEV strain HB 171 (6 positive of 6 surviving inoculated animals), CGl 223 (6/12), Battaune 17/H9 (8/8), HM 4-2 (6/6), Neudörfl (4/6), or BaWa 15/943 (1/6). In contrast to this, the corresponding serum sample tested negative in most cases (29 out of 31, Table 2, Figures 1–3 and Figure 5).

Brain samples also tested positive for TBEV by RT-PCR in considerable amounts, and mostly correlated with the detection of positive whole blood samples (9 out of 31) (Table 2, Figures 1–3 and 5). The spine samples tested positive in 6 out of the 31 viremic voles. In addition to these 31 animals, viral RNA was detected in 2 further voles, namely in the brain and spine sample of animals inoculated with the Scharl strain (2/12) (Table 2, Figure 2b).

**Table 2.** Results of RT-PCR testing of all samples taken from inoculated bank voles at 28 days post infection. Groups with at least one positive sample are shaded in grey. S.int. and l. int.: small and large intestine.

| Virus Strain | Bank Vole Lineage | Number of Positive Samples/Total Number | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Blood | Serum | Brain | Spine | Lung | Heart | s. int. | l. int. | Liver | Spleen | Kidney | Bladder | Uterus | Faeces | Urine | Lavage |
| HM 4-2 | Western | 6/6 | 1/6 | 3/6 | 3/6 | 0/6 | 0/6 | 2/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 [1] | 0/6 | 0/6 | 0/6 |
| BaWa 15/943 | Western | 1/6 | 0/6 | 1/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/4 | 0/6 |
| Neudörfl | Western | 4/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/2 | 0/6 |
| HB 171/11 | Western | 6/6 | 0/6 | 1/6 | 0/6 | 1/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 1/6 | 0/6 | 0/6 | 0/6 | 0/5 | 0/6 |
| Langat virus | Western | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 [1] | 0/5 | 0/1 | 0/6 |
| Battaune 17-H9 | Western | 3/3 | 0/3 | 2/3 | 1/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/1 | 0/3 |
| | Carparthian | 5/5 | 0/5 | 2/5 | 2/5 | 0/5 | 2/5 | 1/5 | 2/5 | 0/5 | 1/5 | 0/5 | 0/5 | 0/5 | 0/4 | 0/1 | 0/5 |
| CGl 223 | Western | 2/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/2 | 0/6 |
| | Carparthian | 4/6 | 1/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 1/6 | 0/6 | 0/6 | 0/6 | 0/5 | 0/1 | 1/6 |
| IZ58 | Western | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 [1] | 0/6 | 0/2 | 0/6 |
| | Carparthian | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/0 | 0/6 |
| Scharl | Western | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/2 | 0/6 |
| | Carparthian | 0/6 | 0/6 | 2/6 | 2/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 |
| Σ | Σ | 31/74 | 2/74 | 11/74 | 8/74 | 1/74 | 2/74 | 3/74 | 2/74 | 0/74 | 2/74 | 1/74 | 0/74 | 0/74 | 0/71 | 0/33 | 1/74 |

[1] one animal was male; therefore, a testicle was sampled instead of the uterus.

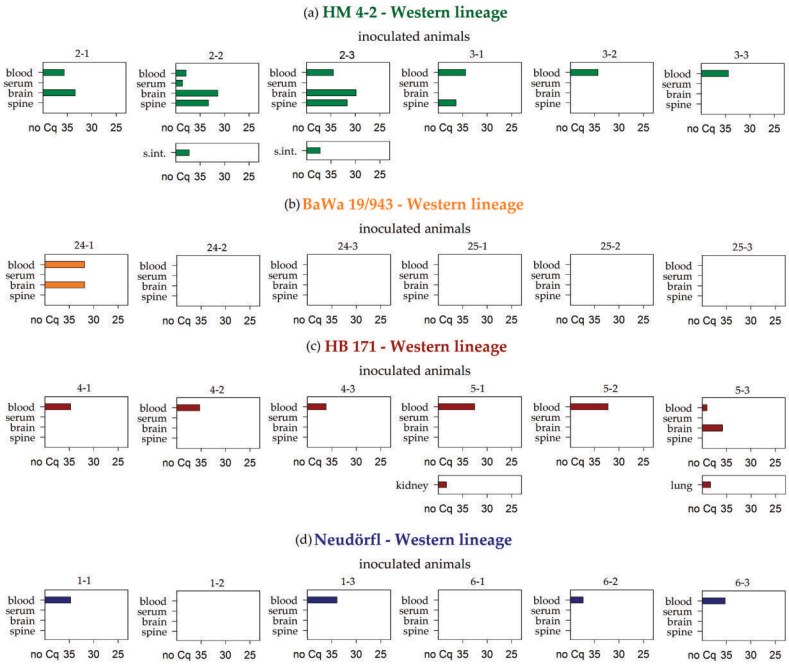


**Figure 1.** RT-PCR results of blood, serum, brain, and spine samples for the bank voles of the Western lineage that were inoculated with the (**a**) HM 4-2, (**b**) BaWa 15/943, (**c**) Neudörfl, and (**d**) HB 171 TBEV-Eu strains. Further additional positive samples are listed per animal. Measures are given in quantification cycle values (Cq). S.int.: small intestine.

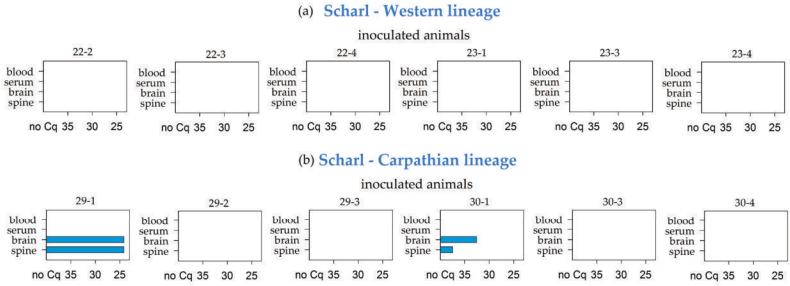


**Figure 2.** RT-PCR results of blood, serum, brain, and spine samples for the bank voles of the Western (**a**) and Carparthian (**b**) lineages that were inoculated with the Scharl TBEV-Eu strain. Measures are given in quantification cycle values (Cq).

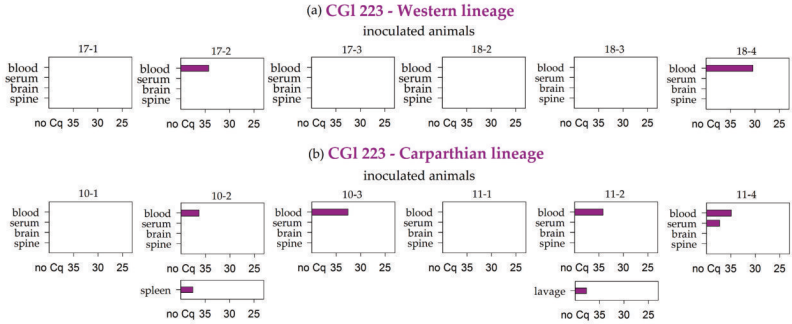


**Figure 3.** RT-PCR results of blood, serum, brain, and spine samples for the bank voles of the Western (**a**) and Carparthian (**b**) lineage that were inoculated with the CGI 223 TBEV-Eu strain. Further additional positive samples are listed per animal. Measures are given in quantification cycle values (Cq).

No animal inoculated with the strain IZ58, which originated from an area not endemically affected, tested positive for any examined sample. The animals inoculated with LGTV likewise tested negative in all of the analyzed samples (Table 2).

Interestingly, Battaune 17-H9 was the only strain that caused premature losses in both bank vole lineages. All samples of the animals that were euthanized at 3, 5, or 6 dpi tested positive in the RT-PCR, and only the brain sample from the animal that died at 3 dpi remained negative. In the animal that was prematurely euthanized at 12 dpi, viral RNA was detected in the whole blood, the brain, and the spine, as well as in the digestive tract samples (Figure 4).

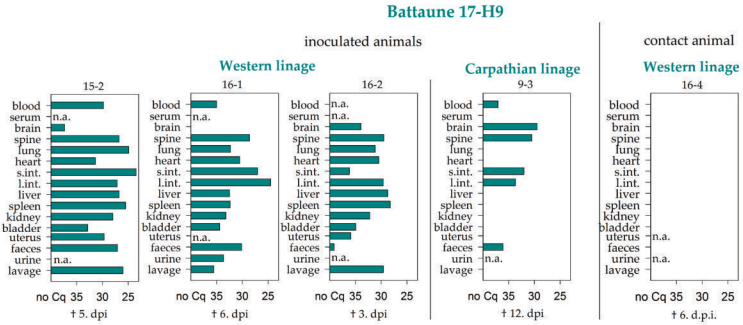


**Figure 4.** RT-PCR results of the bank voles that had to be taken out prior to the endpoint. The day of removal is given underneath the respective graph as days post infection (d.p.i). Missing samples are marked with n.a. (not available).

The remaining animals that were inoculated with the Battaune 17-H9 strain also resulted positive for the whole blood samples independently of the vole evolutionary lineage. In four voles, two of each linage, the brain sample was also positive, and the corresponding spine samples tested positive in three out of these four cases (Figure 5). Every in-contact and environmental control animal tested negative by RT-PCR.

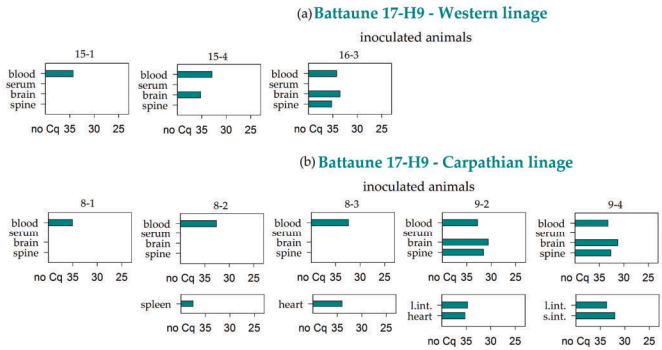


**Figure 5.** RT-PCR results of blood, serum, brain, and spine samples for the bank voles of the Western (**a**) and Carpathian (**b**) lineages that were inoculated with the Battaune TBEV-Eu strain. Further additional positive samples are listed per animal. Measures are given in quantification cycle values (Cq). S.int. and l. int.: small and large intestine.

### 3.3. Comparison of Viral RNA Detection and Cell-Culture Infectivity

All eight TBEV-Eu strains showed a mathematical correlation between Cq value and logarithmic $TCID_{50}$/mL value. The higher the $TCID_{50}$/mL value, the earlier viral RNA was detected via RT-PCR, leading to lower Cq values. Scatter plot visualization showed a clustering of Cq values in accordance with $TCID_{50}$/mL values and a single linear regression line for all TBEV-Eu isolates. Further RT-PCR managed to detect viral RNA even in dilutions with a negative $TCID_{50}$/mL value (Figure 6).

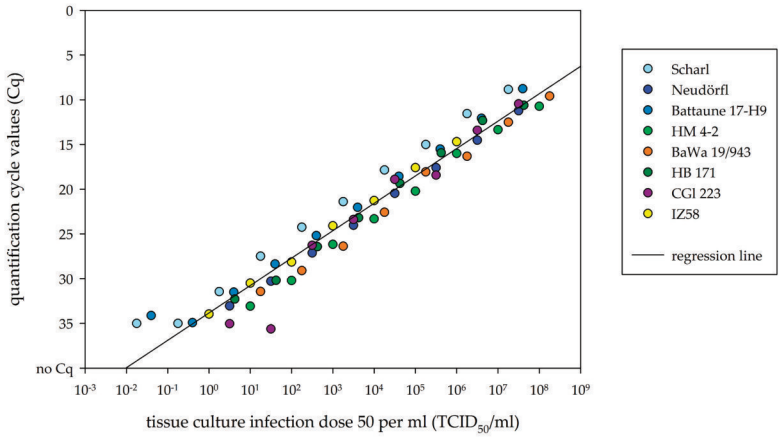


**Figure 6.** Scatter plot between logarithmic tissue culture infection dose 50 ($TCID_{50}$)/mL and quantification cycle values (Cq) of TBEV-Eu isolates. Dots are color marked in accordance to each TBEV-Eu isolate. Regression line is drawn for the mean of all plots.

The RT-PCR results of the tested tissue samples showed Cq values from around 25 to 35. Estimating the infectivity on cell cultures from the single regression line leads to a $TICD_{50}$/mL of $10^{1.37}$ for a Cq value of 30. The Cq values 25 and 35 led to $TCID_{50}$/mL values of $10^{2.92}$ and $10^{-0.18}$, respectively.

### *3.4. Virus Isolation*

Virus isolation was attempted from all positive brain and spine samples, as well as from selected positive organ samples of the bank voles that died prematurely. Virus was successfully reisolated from the brain tissue of two of the bank voles that had been inoculated with the HM 4-2 strain (animals 2-2 and 2-3). From the prematurely euthanized bank voles, TBEV-Eu was reisolated from one large intestine sample (16-1), one heart sample (16-1), and one lung sample (15-2). Virus isolation from positive EDTA blood samples was attempted with the samples that had the lowest Cq values, but failed due to the pronounced cell toxicity of the samples.

### *3.5. Comparative Antibody Detection between Sera and Lavages*

In the serum samples of all surviving inoculated animals, specific neutralizing antibodies could be detected at the end of the study (28 dpi), while neither the in-contact animals nor the voles that were used as environmental controls seroconverted. Overall, all strains led to high values of neutralizing antibodies in the inoculated bank voles of the Western lineage as well as those of the Carpathian lineage (Tables 3 and 4).

There were no striking differences between the bank voles of the two lineages when inoculated with the same TBEV-Eu strain. For details, see Table 4.

The comparative testing of both serum samples and lavage samples showed no direct correlation. However, the values using the lavage samples were always markedly lower than the values using the corresponding serum sample. In eight lavage samples, no neutralizing antibodies were detected even though the corresponding serum sample showed a neutralization titer of at least 1:20. The neutralizing titers of all tested lavage samples did not exceeded 1:40. Only 11 out of the 78 tested lavage samples had neutralizing titers of more than 1:10. In comparison to this, 48 out of the 74 available serum samples reached neutralizing titers of 1:120 or higher (Tables 3 and 4).

**Table 3.** Results of the microneutralization assay comparing the usage of lavage samples to serum samples for the animals that had been inoculated with either the Neuddörfl, HM 4-2, HB171/11, or BaWa 15/943 TBEV-Eu strain or Langat virus. In-contact animals are shaded in grey. $ND_{50}$: 50% neutralizing dose.

| | HM 4-2 | | | BaWa 15/943 | | | Neudörfl | |
|---|---|---|---|---|---|---|---|---|
| ID | serum | lavage | ID | serum | lavage | ID | serum | lavage |
| | $ND_{50}$ | $ND_{50}$ | | $ND_{50}$ | $ND_{50}$ | | $ND_{50}$ | $ND_{50}$ |
| 2-1 | 1:960 | 1:5 | 24-1 | 1:480 | 1:10 | 1-1 | 1:1280 | 1:5 |
| 2-2 | 1:240 | neg. [1] | 24-3 | 1:240 | 1:5 | 1-2 | 1:640 | 1:10 |
| 2-3 | 1:1280 | 1:7.5 | 24-4 | 1:320 | 1:20 | 1-3 | 1:240 | 1:5 |
| 2-4 | neg. [1] | neg. [1] | 24-2 | neg. [1] | neg. [1] | 1-4 | neg.[1] | neg. [1] |
| 3-1 | 1:1280 | 1:40 | 25-1 | 1:80 | 1:5 | 6-1 | 1:960 | 1:10 |
| 3-2 | 1:1280 | 1:40 | 25-2 | 1:120 | 1:5 | 6-2 | 1:1280 | 1:7.5 |
| 3-3 | 1:1280 | 1:10 | 25-3 | 1:160 | 1:15 | 6-3 | 1:1280 | 1:30 |
| 3-4 | neg. [1] | neg. [1] | 25-4 | neg. [1] | neg. [1] | 6-4 | neg. [1] | neg. [1] |
| | HB 171/11 | | | Langat virus | | | | |
| ID | serum | lavage | ID | serum | lavage | | | |
| | $ND_{50}$ | $ND_{50}$ | | $ND_{50}$ | $ND_{50}$ | | | |
| 4-1 | 1:640 | 1:10 | 26-1 | 1:120 | neg. [1] | | | |
| 4-2 | 1:1280 | 1:40 | 26-2 | 1:160 | 1:2.5 | | | |
| 4-3 | 1:640 | 1:5 | 26-3 | 1:240 | 1:2.5 | | | |
| 4-4 | neg. [1] | neg. [1] | 26-4 | neg. [1] | neg. [1] | | | |
| 5-1 | 1:160 | 1:2.5 | 27-1 | 1:160 | neg. [1] | | | |
| 5-2 | 1:1280 | 1:5 | 27-3 | 1:320 | 1:10 | | | |
| 5-3 | 1:1280 | 1:40 | 27-4 | 1:320 | 1:7.5 | | | |
| 5-4 | neg. [1] | neg. [1] | 27-2 | neg. [1] | neg. [1] | | | |

[1] neg. stands for a detection limit of <1:20 for serum samples and <1:5 for lavage samples.

**Table 4.** Results of the microneutralization assay comparing the usage of lavage samples to serum samples for the Battaune 17-H9, CGl 223, IZ58, and Scharl strains. The bank voles of the Western linage are compared to the bank voles of the Carpathian lineage. Contact animals are shaded in grey.

| Battaune 17-H9 | | | | | | CGl 223 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Western lineage | | | Carpathian lineage | | | Western lineage | | | Carpathian lineage | | |
| ID | serum $ND_{50}$ | lavage $ND_{50}$ | ID | serum $ND_{50}$ | lavage $ND_{50}$ | ID | serum $ND_{50}$ | lavage $ND_{50}$ | ID | serum $ND_{50}$ | lavage $ND_{50}$ |
| 15-1 | 1:40 | 1:10 | 8-1 | 1:160 | 1:2.5 | 17-1 | 1:60 | 1:2.5 | 10-1 | 1:30 | 1:7.5 |
| 15-2 | n.a. [2] | 1:10 | 8-2 | 1:120 | neg. [1] | 17-2 | 1:80 | 1:10 | 10-2 | 1:80 | 1:10 |
| 15-4 | 1:240 | 1:10 | 8-3 | 1:40 | 1:5 | 17-4 | 1:160 | 1:2.5 | 10-3 | 1:20 | neg. [1] |
| 15-3 | neg. [1] | neg. [1] | 8-4 | neg. [1] | neg. [1] | 17-3 | neg. [1] | neg. [1] | 10-4 | neg. [1] | neg. [1] |
| 16-1 | n.a. [2] | 1:20 | 9-2 | 1:80 | 1:10 | 18-2 | 1:160 | 1:7.5 | 11-1 | 1:40 | 1:5 |
| 16-2 | n.a. [2] | 1:10 | 9-3 | n.a. [2] | 1:15 | 18-3 | 1:160 | 1:5 | 11-2 | 1:160 | 1:5 |
| 16-3 | 1:160 | 1:15 | 9-4 | 1:240 | 1:2.5 | 18-4 | 1:160 | 1:5 | 11-4 | 1:320 | neg. [1] |
| 16-4 | neg. [1] | neg. [1] | 9-1 | neg. [1] | neg. [1] | 18-1 | neg. [1] | neg. [1] | 11-3 | neg. [1] | neg. [1] |
| IZ58 | | | | | | Scharl | | | | | |
| Western lineage | | | Carpathian lineage | | | Western lineage | | | Carpathian lineage | | |
| ID | serum $ND_{50}$ | lavage $ND_{50}$ | ID | serum $ND_{50}$ | lavage $ND_{50}$ | ID | serum $ND_{50}$ | lavage $ND_{50}$ | ID | serum $ND_{50}$ | lavage $ND_{50}$ |
| 19-2 | 1:120 | 1:10 | 12-2 | 1:20 | 1:2.5 | 22-2 | 1:240 | 1:7.5 | 29-1 | 1:40 | 1:5 |
| 19-3 | 1:120 | 1:7.5 | 12-3 | 1:20 | neg. [1] | 22-3 | 1:320 | 1:5 | 29-2 | 1:60 | 1:2.5 |
| 19-4 | 1:20 | 1:5 | 12-4 | 1:40 | 1:5 | 22-4 | 1:120 | 1:5 | 29-4 | 1:80 | 1:7.5 |
| 19-1 | neg. [1] | neg. [1] | 12-1 | neg. [1] | neg. [1] | 22-1 | neg. [1] | neg. [1] | 29-3 | neg. [1] | neg. [1] |
| 20-1 | 1:30 | 1:5 | 13-1 | 1:40 | 1:2.5 | 23-1 | 1:160 | 1:7.5 | 30-1 | 1:80 | neg. [1] |
| 20-2 | 1:40 | 1:10 | 13-3 | 1:40 | 1:15 | 23-3 | 1:120 | 1.7.5 | 30-3 | 1:60 | 1:2.5 |
| 20-3 | 1:20 | 1: 5 | 13-4 | 1:80 | neg. [1] | 23-4 | 1:320 | 1:5 | 30-4 | 1:20 | neg. [1] |
| 20-4 | neg. [1] | neg. [1] | 13-2 | neg. [1] | neg. [1] | 23-2 | neg. [1] | neg. [1] | 30-2 | neg. [1] | neg. [1] |

[1] neg. stands for a detection limit of <1:20 for serum samples and <1:5 for lavage samples. [2] Missing samples are marked with n.a. (not available).

## 4. Discussion

TBEV is one of the most important tick-transmitted zoonotic pathogens [56] and can lead to severe meningoencephalitis in humans [15]. The virus is endemic in forest and grassland areas, where it is transmitted to a multitude of animal species. Among them, small mammalians are suspected to be of importance for TBEV circulation, enabling the virus to be spread among the tick population [57]. To better understand the interaction between TBEV and its putative natural hosts, the virus–host interaction was studied under experimental conditions using European strains of TBEV in Central and Carpathian European voles.

In the present study, all TBEV-Eu strains used led to successful infection in all inoculated bank voles, as demonstrated by the detection of viral RNA and/or the presence of neutralizing antibodies. TBEV-Eu genome was found after 28 days in the whole blood samples of all bank voles that were inoculated with either HM 4-2 or HB 171/11, as well as in four out of six bank voles that were inoculated with the Neudörfl strain, suggesting a long-lasting viremia of at least up to a month. In addition, viral RNA was detected in the brain samples of numerous animals. The strain HM 4-2 was even successfully reisolated in cell culture from two positive brain samples, proving that indeed infectious virus was still present in the bank voles at 28 days post infection. For the common vole (*Microtus arvalis*), it was shown that this persistent infection in the central nervous system can potentially last for 100 days [37], which should be further explored for the bank vole.

In comparison to TBEV-Eu, the closely related, serologically cross-reactive LTGV was used as a control. This virus also belongs to the tick-transmitted Flaviviridae complex and leads to occasional meningoencephalitis in humans, but is only endemic in Malaysia. [43]. All inoculated bank voles became infected when inoculated with LGTV, which was proven by the presence of neutralizing

antibodies, but no viral RNA was detected in any samples through RT-PCR testing. This was in clear contrast to the persistent brain infection and viremia in bank voles inoculated with TBEV-Eu strains and, therefore, may indicate an efficient adaptation of the TBEV-Eu strains to the locally occurring small mammalian host.

However, the most striking result of this study was the detection of viral RNA in the whole blood sample of inoculated animals 28 days after infection, while the corresponding serum sample remained negative in most cases. This phenomenon was previously hinted at in a study conducted in the 60s [36], an experimental study of TBEV-Sib in the red vole (*Myodes rutilus*) [58], and in a trapping study that differentiated between serum and blood clots [40]. Nevertheless, this fact is often overlooked and can lead to false assumptions concerning the duration of potential viremia [39] and an underestimation of prevalence. Since TBEV was only found in the whole blood samples and not in the corresponding serum samples, TBEV most likely attaches to or infects some type of blood cell, and potentially remains there for at least 28 days in infected bank voles. A study by Krylova et al. [59] examined the interaction of different pathogenic strains with human blood samples in the first day after infection. A highly pathogenic strain of the TBEV-FE subtype showed rapid penetration and active reproduction in the blood cells, while a lowly pathogenic strain remained almost entirely in the serum fraction [59]. Thus, the interaction with the blood cells seems to contribute to the pathogenicity of TBEV. In addition to this, it is quite interesting that TBEV can remain in blood cells for a duration of 28 days despite the presence of neutralizing antibodies.

TBEV is known to rearrange intracellular cytoplasmic compartments in order to replicate in them, and these compartments are supposed to be inaccessible for the host immune system [60,61]. The antibodies circulating in the serum fraction of the blood might neutralize TBEV virions released from infected cells, but do not interfere with replication in the intracellular cytoplasmic compartments. Furthermore, the potential infection of naïve ticks is most likely not hindered by the presence of neutralizing antibodies [62], since co-feeding supposedly works through the transmission of infected cells [63]. One of the cell fractions infected during the co-feeding process is monocytes [63], and their interaction with TBEV has been well studied. They become infected with TBEV, show a multitude of structural changes in reaction to it [64], and can successfully transmit TBEV to laboratory mice [65]. Therefore, monocytes, the progenitor cells of macrophages, might be the location of replication of TBEV. However, since the findings of the present study were quite unexpected, the whole blood samples were frozen for RT-PCR testing and, therefore, the isolation of different cell fractions was not possible. Thus, the interaction of the virus with the host blood cells of the potential reservoir species bank vole should be part of future investigations.

Four TBEV-Eu strains were simultaneously inoculated in two different evolutionary bank vole lineages to assess the influence of the vole origin when inoculated with virus strains isolated in areas where only one of both lineages naturally occurs. Some bank voles that were inoculated with the Battaune 17-H9 strain had to be euthanized prematurely, independently of the vole lineage. One of the voles died spontaneously, but did not display any neurological symptoms. Two additional bank voles of the Western lineage and one of the Carparthian lineage were euthanized within 12 days. Since one of the in-contact animals as well as one environmental control animal had to be taken out of the experiment prematurely, these early loses cannot be conclusively interpreted as being result of the TBEV infection, especially since the control animals tested negative by RT-PCR. However, the high viral RNA loads in nearly all organ samples of the inoculated bank voles strongly hinted at the involvement of TBEV in the death of one bank vole and the rapid weight loss of the other three inoculated animals. The reasons for the divergent behavior of this virus strain in comparison to the other strains used in the present study remain unknown, and additional animal experiments need to be performed to substantiate this phenomenon; however, the vole lineage did not appear to play a role. All of the bank voles of the Western as well as of the Carparthian lineage of the infection group that reached the endpoint of this study showed an RNAemia of at least 28 days. The virus strain Battaune 17-H9, which did not show any prominent amino acid substitutions in the envelope gene (data not shown)

potentially leading to increased virus virulence, was isolated in Leipzig, Germany, where the Western vole linage is dominant [45,66]. Since bank voles of the Carpathian lineage showed a similar infection pattern, it seems that they are able to take on the role of their Western counterpart, which could be confirmed by using further strains. The strain CGl 223 was detected in the whole blood samples of some bank voles from both lineages, and the respective brain samples tested consistently negative. CGl 223 was isolated from the Slovak Republic, where the Carpathian vole lineage is primarily found [66]. Again, similar results for both lineages do not support an influence of different lineages on the TBEV transmission cycle.

The strain IZ58, which was isolated from a region where TBEV is not considered to be endemic [47], led to no detection of viral RNA in either bank vole lineage at 28 dpi. A difference between the two lineages was only seen for the strain Scharl, which was originally isolated from the brain of a human. While all bank voles of the Western lineage remained negative in all samples, the brain as well as the spine samples of two of the bank voles of the Carpathian lineage were positive in the RT-PCR testing; however, clinical signs were not observed in any of the animals. Thus, the overall results of both vole lineages were quite similar for all simultaneously tested strains, which speaks against an influence of different lineages on the interaction between TBEV and its natural rodent host. With regard to virus transmission between the rodent hosts, it is highly unlikely that TBEV-Eu is transmitted horizontally, since none of the in-contact animals seroconverted, although the viral load seemed to be immense in the first week after infection and virus was successfully reisolated from selected organs. However, previous studies have described horizontal and vertical transmission between red voles when infected with a TBEV-Sib strain [58].

The animals that had to be euthanized early hinted at a systemic infection in the first week, with a neuroinvasion between days three and five. A week later, viral RNA was only detected in the whole blood samples, the brain/spine samples, and, surprisingly, the samples of the digestive tract. In line with that, TBEV has only recently been tentatively linked with gastrointestinal symptoms in humans [46]. Furthermore, humans can become infected with TBEV through the consumption of non-pasteurized dairy products [67], which indicates at least some degree of susceptibility of the gastrointestinal tract for TBEV infection.

To relate the generated real-time PCR data to actual infectivity in cell culture, comparative analysis was performed. Overall, RT-PCR led to the detection of viral RNA in virus dilutions with a $TCID_{50}$/mL as low as $10^{-1.75}$. This finding suggests that theoretically, even a single viral genome fragment could be detected with the presented RT-PCR. The organ samples collected from the animals that were taken out prior to the endpoint showed lower Cq values, leading to estimated $TCID_{50}$/mL values that ranged from around $10^{1.37}$ to $10^{2.92}$. In accordance, virus reisolation on cell culture was successful. The viral genome that was detected 28 dpi, mainly in the brain samples, only correlated to $TCID_{50}$/mL values of around $10^{-0.18}$ to $10^{1.37}$, complicating the reisolation in cell culture. Therefore, viral infectivity seems to decrease over the course of infection. However, Cq values of whole blood samples taken 28 dpi were comparable to the Cq values of whole blood samples from the animals that were taken out 5, 6, and 12 dpi, hinting at a consistent viremia throughout the course of 28 days. Cq values from the whole blood samples resulted from an extraction volume of 15 µL instead of the 100 µL that was used for organ samples and virus dilutions. Therefore, infectivity on cell culture may be even higher than estimated by this comparative analysis. To confirm this first estimation, additional experiments are needed in this now established animal model, investigating earlier time points in the course of infection of TBEV in bank voles.

In addition to the characterization of the virus–host interaction of different TBEV-Eu strains in the bank vole, the suitability of chest cavity lavage as a diagnostic material to detect neutralizing antibodies was investigated, since serum samples are not always available when animals die a natural death. Furthermore, such lavages are frequently used in epidemiological studies of wild caught animals when serum is not available [45,68]. The comparative testing of both sample matrices, i.e., serum and chest cavity lavage, showed that the chest cavity lavage does principally enable the detection of neutralizing

antibodies. However, the values were far lower than the values that were detected in the serum samples of the same animal, which led to false negative results in seven bank voles. Therefore, the use of such lavage samples is convenient when no serum sample is available, but should be considered with caution for epidemiological studies due to its reduced sensitivity. For such studies, additional sample matrices should be validated to offer a reliable alternative to serum samples.

## 5. Conclusions

TBEV-Eu appears to be well adapted to the bank vole host, leading to long-lasting viremia and an infiltration of the brain without causing visible neurological symptoms. These findings fully support the role of bank voles as a reservoir host for TBEV, and encourage further research on this topic.

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# 5. Discussion

**Interaction between TBEV and its natural mammalian host**

The interplay between vector-transmitted flaviviruses and their natural hosts is hardly studied, since wild and free-living animals are not easy to handle and the keeping under laboratory conditions is often combined with strict requirements concerning housing and supervision (Jackson 1997). In this study, bank voles were chosen as a representative of the natural mammalian hosts of TBEV in Europe, since they show high antibody prevalence rates in various TBEV surveillance studies and have been long suspected to act as reservoir hosts for TBEV (Michelitsch, Wernike et al. 2019). Furthermore, there are outbreed colonies of different bank vole lineages available. They provide bank voles that are born in captivity and therefore are accustomed to the living conditions in a laboratory faculty. This circumvents the stress wild bank voles would have to experience when being caught in the wild and brought to the confinements of a laboratory facility (Turner and Paterson 2013). Since the bank voles originate from an outbreed line, their genetic diversity is not lost and can still be seen as a model for wild-living bank voles (Brekke, Steele et al. 2018).

A TBEV infection model was successfully established, which allowed the *in vivo* characterization of eight TBEV-Eu strains as well as LGTV over the course of four weeks. This first screening of TBEV-Eu strains showed that bank voles have the potential to be a reservoir host for TBEV as it is indicated by the long-lasting viremia. The detection of viral RNA in whole blood samples 28 dpi exceeds the previously described viremia of three to nine days by far (Ernek, Kožuch et al. 1963). This might be attributed to improved detection methods as well as to the choice of sample material. Even back in 1966, Heigel and Zeipel noted that virus could be detected in the blood cell fraction longer than in the corresponding plasma fraction (Heigl and Zeipel 1966). However, in a more recent study serum samples were used overlooking the importance of examining the blood cell fraction, and an occasional viremia for up to 14 days was described in bank voles after the infection with a TBEV-Eu strain. Knowing this dependence from the sample material, the detection of TBEV-Fe for 25 days and TBEV-Sib for 84 days in a single animal each is explainable and hints at an even longer lasting blood-cell-bound viremia (Tonteri, Kipar et al. 2013). The absence of viral TBEV RNA in the serum samples in combination with the detection of viral RNA in the corresponding whole blood samples,

that was seen in the presented study, hints at the infection of some kind of cellular component of the blood. Previous studies have shown that TBEV infects and replicates in human blood leukocytes (Krylova, Smolina et al. 2015). Among them especially monocytes are suspected to be the targeted cells (Plekhova, Pustovalov et al. 2017). Future studies should therefore also focus on the identification of the infected blood cell types.

In the presented animal trial, bank voles were mostly clinically unaffected by the infection, since the majority of animals showed no signs of disease. Only the strain Battaune 17-H9, which was originally isolated from the brain of a bank vole, caused premature fatalities. The animals that were euthanized 3, 5 and 6 dpi were PCR-positive in nearly all analyzed organs. In the bank vole that was euthanized 12 dpi, viral RNA was found in the brain and spine but also in the samples of the intestinal tract. The infection of the gastrointestinal tract in combination with the positive feces samples that were detected in all of these four animals, hints at a possible virus shedding at least for bank voles that are infected with the strain Battaune 17-H9. In the light of these results, an alimentary infection of naïve bank voles might be possible, since this route of transmission is known to take place in humans (Balogh, Egyed et al. 2012). Nevertheless, a horizontal spread of TBEV-Eu among the bank vole population is still very unlikely, since none of the in-contact animals that were housed together with TBEV-Eu infected bank voles showed any signs of contact with TBEV, as determined by real-time PCR and neutralization assays.

The *in vivo* characterization of different TBEV strains in bank voles revealed differences regarding the virus dynamics within the infected hosts. LGTV, which is only endemic in Malaysia (Smith 1956) and is a lowly pathogenic virus that is similar to TBEV in its transmission cycle, was used for comparison. As expected, the inoculated bank voles became successfully infected, as shown by the detection of neutralizing antibodies. However, viral RNA was not found at 28 dpi. Strain IZ58 showed the same picture. As this strain was isolated from a region (Apitzsch, Sinnecker et al. 1968) that was categorized by the local authority as a no risk area (Robert Koch-Institut 2019), the lack of adaption to the natural reservoir host population is conceivable. The isolation of this strain in 1968 might have been an isolation by chance from a tick that was brought into this area by a traveling bird and did not find the ecological conditions to establish a new endemic region (Hasle 2013). In contrast to the two aforementioned virus strains, the TBEV strains HM 4-2, Battaune, HB 171 and BaWa 19/933

led to the development of viremia and infiltration of the brain. While this was the case for nearly all animals inoculated with the former three, BaWa 19/933 was only detected in one of the inoculated bank voles. The strains Neudörfl and CGI 223 led to the development of viremia in most infected animals.

Overall, the virus dynamics of the TBEV strains in the newly developed infection model showed striking differences between the strains. The development of a long lasting viremia and especially the infiltration of the brain following the inoculation of certain TBEV strains are two findings that may help determining the virulence of TBEV strains in the future. As it is discussed in the following, the virulence of TBEV strains is mostly defined by parameters of neuropathogenicity, which are usually determined by using laboratory mice models (Mandl 2005). Therefore, *in vivo* characterization studies comparing different TBEV strains rely on lethal dose testing and require a different testing method for single parameters of neuropathogenicity (Leonova, Belikov et al. 2017).

The neuropathogenicity of TBEV is characterized by its neuroinvasiveness and neurovirulence. Neuroinvasiveness describes the ability of the virus to enter the CNS (Mandl 2005). Viruses usually enter the CNS either though neuronal transport from the periphery or by crossing the barriers that circumvent the CNS, like the BBB, which can be achieved by numerous ways (Spindler and Hsu 2012). Flaviviruses are described to enter the CNS through passage of the BBB by infecting the brain microvascular endothelial cells (BMECs). These infected BMECs than either release infectious particles themselves or downregulate the proteins that make up their tight junctions, by which virus particles are enabled to cross the BBB in between the BMECs (Mustafá, Meuren et al. 2019). The mechanism by which TBEV crosses the BBB is not yet fully understood. Although BBB integrity is compromised during TBEV infection, it does not seem to be a necessity for the initial entrance into the CNS (Růžek, Salát et al. 2011). The lack of clinical symptoms in combination with the infection of some kind of cellular blood component might hint at a circumvention of the BBB for TBEV neuroinvasion at least in bank voles. A similar effect is known for Zika virus, where infected monocytes enter the CNS in a process that is called 'Trojan Horse' (Ayala-Nunez, Follain et al. 2019, de Carvalho, Borget et al. 2019).

Once the virus entered the CNS, it starts to replicate in neuronal cells. TBEV primarily targets large neurons of various brain regions (Velay, Paz et al. 2019). The damage that is done to the CNS when the virus has entered, is described as neurovirulence (Mandl 2005). It is not entirely

clear, if TBEV damages the CNS by a direct cytopathogenic effect, or if the majority of damage is done by the induced immune response (Velay, Paz et al. 2019). Both of the described neuropathogenicity mechanism are determined by using animal models. These models are established in laboratory mice, since they are highly susceptible to TBEV infection (Mandl 2005) and are easier to handle and breed than animal species that are not accustomed to laboratory conditions (Jackson 1997). Standard laboratory mice strains, like BALB/c and Swiss Albino, develop severe neurological sings that often end lethal. While neuroinvasion is tested by peripheral inoculation, neurovirulence is determined by inoculating juvenile mice intracranially with TBEV. Since wild-type TBEV strains cause lethal infections in both tests, parameters like the time until onset of symptoms are considered.

Neuropathogenicity in laboratory mice is described to be induced with a very high efficacy, so that the titration of some TBEV strains may stop being lethal only after no infectious particles can be detected in the inoculum by cell-culture based methods (Mandl 2005). Mortality after peripheral TBEV injection also does not follow a usual dose-response curve, even when inbred mice are used (Hayasaka, Nagata et al. 2009). In this context, it would be interesting to see if the selective neuroinvasion of different TBEV strain that is seen in bank voles can be aligned with the actual neurovinvasion that is seen in humans. For example, strain HB171/11 is described to be low-pathogenic in humans, while the characterization in the common mouse model did result in a reduced lethality of 60% and a delayed neuroinvasion in comparison to the highly virulent strain Torö-2003 (Kurhade, Schreier et al. 2018). In the presented study, HB171/11 showed a viremia in all inoculated bank voles and neuroinvasion in one (Michelitsch, Tews et al. 2019). None of the bank voles showed any clinical signs, or had to be euthanized prematurely. The *in vivo* characterization of the strain Neudörfl that is generally described as a low-virulence reference strain, leads to a survival rate of 20% in mice after peripheral inoculation when followed-up for 28 days (Wallner, Mandl et al. 1996). Again, bank voles that were inoculated with the strain Neudörfl showed a survival rate of 100% when kept for 28 days. The infection model in the natural host might therefore be more descriptive of the virulence and neuropathogenicity of different TBEV strains than the common laboratory mouse model or at least offers a different perspective, also on the reservoir host features.

A recent study in Western Siberia has shown that the local dominate species, the northern red-backed vole (*Myodes rutilus*) and the field mice (*Apodemus agrarius*), show differences in

the interaction with TBEV. The northern red-backed vole seems to exceed the field mouse in terms of virus susceptibility as well as viral persistence. Accordingly, natural infection rates in northern red-backed voles are significantly higher than in field mice (Morozova, Panov et al. 2020).

The study presented in this thesis has its focus on the situation in Europe, where bank voles and yellow-necked mice are the predominate species. TBEV detection rates in bank voles and yellow-necked mice do not show marked differences in various European surveillance studies (Michelitsch, Wernike et al. 2019). However, overall tick infestation rates are higher in yellow-necked mice than in bank voles (Talleklint and Jaenson 1997) and they are also prone to multiple tick bites, while bank voles develop a resistance after several infestations, at least under laboratory conditions (Dizij and Kurtenbach 1995). Although bank voles develop higher levels of TBEV viremia after tick-bite, yellow-necked mice enable higher rates of TBEV transmission between co-feeding ticks, which exceed those observed in bank voles by four times (Labuda, Nuttall et al. 1993). Therefore, yellow-necked mice may play an equally important role in the TBEV transmission cycle in Europe.

**Influence of regional bank vole lineages on TBEV transmission**

The virus dynamics of TBEV in different bank vole lineages was studies in order to reveal possible differences that might influence the TBEV transmission cycle in nature. A similar connection is already known known for Puumala Orthohantavirus (Drewes, Ali et al. 2017). The majority of testing in this study was performed in bank voles of the Western evolutionary lineage, since this is the dominating lineage in Germany (Filipi, Marková et al. 2015) where the majority of the TBEV strains that were used in this study originated from. Four of these strains were additionally characterized in bank voles of the Carpathian lineage. One of these strains, namely CGl 223, was isolated in Slovakia (Kozuch, Gurycova et al. 1995), where bank voles of the Carpathian lineage are found predominately (Filipi, Marková et al. 2015). The testing revealed no striking differences between the two lineages. Strain CGl 223 was found in whole blood samples of some of the animals of both lineages. The strain Battaune, which was likewise tested in parallel in both bank vole lineages, led to premature loses and was found in whole blood and brain samples of bank voles of the Western as well as the Carpathian vole lineage. The strain Scharl, a human isolate, was found in the brain sample of two bank voles

of the Carpathian lineage, but other than that in none of the bank voles that were inoculated with this strain.

Overall, the selected TBEV strains showed a similar infection dynamics in both lineages. A direct influence of the genetic lineage of the local bank vole population on the TBEV transmission cycle in nature is therefore unlikely. Similar to bank voles, the *Ixodes ricinus* population of Europe is also diverse. At least ten subgroups, based on the analysis of cuticular hydrocarbons, can be differentiated and might be linked to pathogen susceptibility (Estrada-Peña, Daniel et al. 1998). Furthermore, other than the genetic lineages of certain species, the locally occurring small mammalian species might have an influence on the transmission of TBEV, especially on the occurrence of TBEV-subtypes. In Russia and Asia, where TBEV-Sib and TBEV-FE are found predominantly, other small mammalian species seem to take on the role of the bank vole and the yellow-necked mouse (Michelitsch, Wernike et al. 2019).

**Evaluation of TBEV detection methods for surveillance in wild-caught rodents**

TBE is a severe disease that can be prevented by vaccination (WHO-position-paper 2011). Since skepticism against vaccinations in general is on the rise (Tafuri, Gallone et al. 2014), it is important to reliably locate the endemic regions and start targeted vaccination campaigns. TBEV monitoring programs rely on multiple sources. Ticks are collected by flagging (Gäumann, Mühlemann et al. 2010), free ranging life stock is sampled (Klaus, Ziegler et al. 2019) and hunters are compelled to collect samples from game animals (Wurm, Dobler et al. 2000, Duscher, Wetscher et al. 2015, Tonteri, Jokelainen et al. 2016). Still, prevalence studies conducted on rodents have many advantages. Rodents are easy to trap and inhabit most ecological systems. Their home range is small, which helps to determine the range of the actual endemic focus. They are often heavily infested with ticks and they are susceptible to TBEV infection (Achazi, Růžek et al. 2011). A comparative study in a Siberian endemic region showed that TBEV RNA was found in approximately 80 % of trapped rodents and only in about 4% of flagged ticks (Bakhvalova, Chicherina et al. 2016). Projects like the “Rodent-borne pathogens” network collect a multitude of samples from wild rodents all over Germany, which could be used for TBEV surveillance as well (Ulrich, Schmidt-Chanasit et al. 2008).

The presented animal trial (Michelitsch, Tews et al. 2019) allowed to generate defined reference material and to evaluate common practices for its use in TBEV diagnostics. Overall, it became obvious that whole blood is the ideal material for viral RNA detection by real-time RT-PCR. If no blood sample can be collected, brain samples are the second best material where viral RNA can be found even 28 dpi. For the detection of antibodies against TBEV, it seems to be necessary to use actual serum samples in the viral neutralization testing. The usage of lavages of the cavum thoracic is not suitable, since antibody titers are markedly lower in comparison to serum titers and may result in false negative assertions. Moreover, if possible, the antibody prevalence should be preferred to the detection of viral RNA. As it is shown in the presented study, all inoculated bank voles developed high antibody titers. In comparison, the overall number of animals, where viral TBEV was still detected after 28 days, was rather small. When excluding the blood and serum samples, only 31 out of 992 samples were positive for viral TBEV RNA. These samples were collected from only 10 of the 74 (7.4%) inoculated bank voles that reached the end of the study.

In conclusion, whole blood and serum samples should be taken from trapped rodents to reliably detect the ones that had been in contact with TBEV. If this is not possible, false negative results have to be considered.

# 6. Concluding remarks and outlook

This examination of the complex virus dynamics of TBEV-Eu in its natural host, the bank vole, showed that this animal species might take on an important role in the TBEV transmission cycle (Michelitsch, Tews et al. 2019). The detection of viral TBEV in blood and brain samples of various inoculated healthy bank voles hints at a persistent TBEV infection. These animals might be a source of TBEV infection for naïve ticks and therefore would act at least as an amplifying host in the transmission dynamics of TBEV. The screening of multiple TBEV-Eu strains revealed marked differences between these strains in terms of development of viremia and neuropathogenicity that can contribute to a better understanding of the virulence of TBEV strains.

The infection system established in this thesis represents an important step in developing a model of the natural TBEV transmission cycle that will allow the extermination of this complex system under laboratory conditions.

# 7. Summary

Tick-borne encephalitis (TBE) is a vector-borne disease that is present in Europe and the northeastern regions of Asia. It can cause severe neurological symptoms in humans, which can severely limit the quality of life for years of those who are affected. The pathogen causing the disease is the tick-borne encephalitis virus (TBEV). Ticks represent the center of the transmission cycle. They are mainly found in forests and meadow landscapes, where they transmit the virus to all sorts of animal species, including birds and amphibians. Among them, small mammals living on the ground play a special role. They seem to allow the transmission of TBEV within the tick population. Due to the intensive contact with TBEV, wild rodents are also an important tool in the localization of endemic areas.

The bank vole (*Myodes glareolus*) is one of the most common rodents in European forests and occurs in different genetic lineages. In order to better understand the infection dynamics of TBEV in this natural host, experimental studies with European TBEV strains were conducted. Since the testing was carried out in bank voles, which belonged to two different lineages, a genetic influence on the formation of endemic areas was ruled out. The samples obtained were used to compare the possibilities of TBEV detection methods in wild caught rodents in order to better assess conducted epidemiological studies.

Bank voles are well adapted to TBEV. Although brain infiltration has been detected in some animals, there were no neurological symptoms observed. The detection of viral RNA was mainly successful in EDTA whole blood samples, while the corresponding serum samples tested mostly negative. This indicates that TBEV infects cellular components of the blood and thus bypasses neutralization by antibodies. A long-lasting viremia of at least 28 days in some animals could potentially allow transmission of TBEV to naive ticks. These results suggest that bank voles plays an important role in the TBEV transmission cycle as amplifying reservoir hosts.

# 8. Zusammenfassung

Die Frühsommer-Meningoenzephalitis (FSME) ist eine vektorübertragene Krankheit, die in Europa und den nordöstlichen Regionen Asiens vorkommt. Sie kann beim Menschen schwere neurologische Symptome hervorrufen, die die Lebensqualität der Betroffenen jahrelang stark einschränken können. Das krankheitsauslösende Pathogen ist das Frühsommer-Meningoenzephalitis Virus (FSMEV). Es wird von Zecken übertragen, die den Mittelpunkt des Übertragungszyklus darstellen. Zecken sind vor allem in Wald- und Wiesenlandschaften zu finden, wo sie das Virus auf alle dort ansässigen Tierarten übertragen, einschließlich Vögeln und Amphibien. Unter ihnen nehmen kleine, am Boden lebende Säugetieren eine besondere Rolle ein, da sie im Verdacht stehen, die Übertragung von FSMEV innerhalb der Zeckenpopulation zu ermöglichen. Durch den intensiven Kontakt mit FSMEV liefern wildgefangene Nagetiere auch wichtige Hinweise in der Lokalisation von endemischen Gebieten.

Die Rötelmaus (*Myodes glareolus*) ist eine der am häufigsten vorkommenden Nagetierart in europäischen Wäldern und tritt dort in unterschiedlichen genetischen Linien auf. Um die Infektionsdynamik von FSMEV in diesem natürlichen Wirt genauer zu verstehen, wurden experimentelle Studien mit verschiedenen europäischen FSMEV-Isolaten durchgeführt. Da die Testung zum Teil in Rötelmäusen zweier unterschiedlichen Linien erfolgte, konnte ein genetischer Einfluss der Wirte auf die Entstehung von endemischen Gebieten ausgeschlossen werden. Anhand der gewonnenen Proben wurden weiterhin verschiedene Methoden zum Nachweis von FSMEV-Infektionen verglichen, um in der Folge epidemiologische Studien basierend auf Wildfängen besser einschätzen zu können.

Rötelmäuse sind gut an FSMEV angepasst. Sie zeigten für die meisten Stämme keine neurologischen Symptome, obwohl eine Infiltration des Gehirns in einigen Tieren nachgewiesen werden konnte. Der Nachweis von viraler RNA gelang vor allem in EDTA-behandelten Vollblutproben, wobei die entsprechenden Serumproben meist negativ reagierten. FSMEV scheint also zelluläre Komponenten des Bluts zu infizieren und somit eventuell auch die Neutralisation durch Antikörper zu umgehen. Die lang andauernde Virämie von mindestens 28 Tagen in einigen Tieren könnte eine Übertragung von FSMEV auf naive

Zecken ermöglichen. Diese Ergebnisse sprechen für eine zentrale Rolle der Rötelmaus im FSMEV-Übertragungszyklus in Europa.

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# 10. Abbreviations

| | |
|---|---|
| BBB | blood-brain barrier |
| BMECs | brain microvascular endothelial cells |
| CNS | central nervous system |
| Cq | quantification cycle value |
| dpi | days post infection |
| FSME | Frühsommer-Meningoencephalitis |
| FSMEV | Frühsommer-Meningoencephalitis Virus |
| KFDV | Kyasanur Forest disease virus |
| LGTV | Langat virus |
| LIV | louping-ill virus |
| mtDNA | mitochondrial DNA |
| POWV | Powassan virus |
| prM | precursor protein of the M protein |
| RSSE | Russian spring summer encephalitis |
| TBE | tick-borne encephalitis |
| TBEV | tick-borne encephalitis virus |
| TBEV-Bkl | Baikalian subtype of TBEV |
| TBEV-Eu | European subtype of TBEV |
| TBEV-FE | far eastern subtype of TBEV |
| TBEV-Him | Himalayan subtype of TBEV |
| TBEV-Sib | Siberian subtype of TBEV |

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