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TICK-BORNE ENCEPHALITIS VIRUS IN DOGS (*CANIS LUPUS FAMILIARIS*) AND RODENTS OF THE MOUSE (*MURIDAE*) AND HAMSTER (*CRICETIDAE*) FAMILY

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS 7
INTRODUCTION 9
Scientific novelty 10
Aims and objectives of the study 11
1. LITERATURE REVIEW 12
1.1. TBEV taxonomy and structure 12
1.2. Genetic diversity and geographical distribution of TBEV 13
1.3. Vectors of TBEV and biology of *Ixodes ricinus* ticks 14
1.4. TBEV maintenance in nature 17
1.5. Hosts of TBEV 19
1.5.1. Wild animals 19
1.5.2. Domestic animals 22
1.5.3. Birds 24
1.6. Transmission routes and clinical symptoms of TBE 25
1.6.1. TBE in humans 25
1.6.2. TBE in domestic animals 29
2. MATERIALS AND METHODS 31
2.1. Sample collection 31
2.1.1. Dog samples and clinical data 31
2.1.2. Tick samples 31
2.1.3. Small wild rodent samples and sampling sites 32
2.2. Detection of viral RNA 32
2.3. Detection of TBEV-specific antibodies 35
2.3.1. Enzyme-linked immunosorbent assay (ELISA) 35
2.3.2. Virus neutralization test (VNT) 35
2.4. Virus isolation in cell cultures 36
2.5. Viral load quantification 37
2.6. Sequencing and phylogenetic analysis 38
2.7. Statistical analysis 39
2.8. Funding 39
2.9. Ethical statement 39
3. RESULTS AND DISCUSSION 40
3.1. TBEV in dogs 40
3.1.1. The prevalence of TBEV RNA and TBEV-specific antibodies in blood samples of dogs 40
3.1.2. Risk factors of severe course of TBE and lethal outcome in dogs 44

3.1.3. TBEV seroprevalence and relations with clinical symptoms and the prevalence of concurrent tick-borne diseases 45
3.1.4. Virus isolation from PCR-positive dogs' serum and CSF samples 47
3.1.5. Clinical symptoms of TBEV RNA-positive dogs.................................. 47
3.1.6. Clinical symptoms of TBEV-seropositive dogs 50
3.2. TBEV in ticks, collected from dogs..50
3.3. TBEV in wild rodents 52
3.3.1. The prevalence of TBEV RNA in wild rodents..................................... 52
3.3.2. Comparison of TBE viral load in rodent tissue suspensions and isolates in Neuro-2a cells 57
3.3.3. The influence of air temperature on TBEV prevalence in samples of rodent brain and internal organs 60
3.3.4. The prevalence of TBEV in different species of rodents......................... 63
3.3.5. The prevalence of TBEV in fetuses of small wild rodents 67
CONCLUSIONS...69
SUMMARY IN LITHUANIAN 70
REFERENCES...82
LIST OF PUBLICATIONS 109
COPIES OF PUBLICATIONS 110
SUPPLEMENTARY MATERIAL...136
CURRICULUM VITAE 153
ACKNOWLEDGEMENTS 154

LIST OF ABBREVIATIONS

°C	–	degree Celsius
µg	–	microgram
µm	–	micrometer
A. agrarius	–	*Apodemus agrarius* striped field mouse (syn. *Mus agrarius*)
A. flavicollis	–	*Apodemus flavicollis* yellow-necked mouse (syn. *Mus flavicollis*)
A. sylvaticus	–	*Apodemus sylvaticus* wood mouse (syn. *Mus sylvaticus*)
ATCC	–	the American Type Culture Collection
bp	–	base pair
cDNA	–	complementary deoxyribonucleic acid
C. glareolus	–	*Clethrionomys glareolus* bank vole (syn. *Myodes glareolus*)
CNS	–	central nervous system
CSF	–	cerebrospinal fluid
Ct	–	cycle threshold
DNA	–	deoxyribonucleic acid
D. reticulatus	–	*Dermacentor reticulatus* tick
d.p.i.	–	days post-infection
DMEM	–	Dulbecco's Modified Eagle's Medium
ECDC	–	European Centre for Disease Prevention and Control
ELISA	–	enzyme-linked immunosorbent assay
EU	–	European Union
FBS	–	fetal bovine serum
IgG	–	immunoglobulin G
IgM	–	immunoglobulin M
I. pavlovskyi	–	*Ixodes pavlovskyi* tick
I. ricinus	–	*Ixodes ricinus* tick
kb	–	kilobases
M. arvalis	–	*Microtus arvalis* common vole (syn. *Microtus obscurus*)
MEM	–	Modified Eagle's Medium
mg	–	milligram
mL	–	milliliter
M. musculus	–	*Mus musculus* house mouse (syn. *Mus abbotti*)

nm	–	nanometer
nM	–	nanomolar
Nt	–	nucleotide
ORF	–	open reading frame
PBS	–	phosphate-buffered saline
PCR	–	polymerase chain reaction
PES	–	post-encephalitic syndrome
RNA	–	ribonucleic acid
RT-nPCR	–	reverse transcription nested polymerase chain reaction
RT-PCR	–	reverse transcription polymerase chain reaction
TBE	–	tick-borne encephalitis
TBEV	–	tick-borne encephalitis virus
TBEV-Eu	–	tick-borne encephalitis virus of European subtype
TBEV-Sib	–	tick-borne encephalitis virus of Siberian subtype
TBEV-FE	–	tick-borne encephalitis virus of Far East subtype
U	–	unit
VNT	–	virus neutralization test

INTRODUCTION

Tick-borne encephalitis (TBE) is one of Europe and Northern Asia's most common tick-borne zoonotic diseases [1]. The disease is caused by tick-borne encephalitis virus (TBEV), a member of the *Flaviviridae* family [2]. The main transmission route is through the bite of an infected tick, although alimentary transmission following consumption of unpasteurized infected milk products is also possible [3]. EE is medically the most important tick-borne infection which can cause severe neurologic symptoms, long-term sequelae after infection, and death [4].

Although the disease is preventable by vaccination, the incidence of TBE has been increasing during the last few decades [4]. The number of human TBE cases in all endemic regions of Europe has increased by almost 400% in the past 30 years [5]. Moreover, the risk areas have spread and new foci have been discovered [5,6]. The EU/EEA notification rate of human TBE cases for 2016–2018 was 0.6 cases per 100,000 population, increased to 0.7 cases per 100,000 population in 2019, and reached 0.9 cases per 100,000 population in 2020 [5]. The incidence rate of TBE in Lithuania has been one of the greatest in Europe for more than 10 years. According to the National Public Health Centre under the Ministry of Health, the prevalence rate of reported human TBE cases in Lithuania was 58% higher in 2023 compared to 2022 and has increased from 13.3 to 20.8 cases per 100,000 population. The TBE poses a growing health problem in almost all endemic European and Asian countries [4].

Tick-borne encephalitis is a focal infection, therefore the distribution and prevalence of the virus in nature are suggested to be influenced by the interactions between ticks and reservoir hosts of the virus, as well as particular environmental and climatic factors [7–10]. A warming climate has been suggested to be one of the key factors, affecting increasing human TBE incidence [8, 11–14]. Moreover, a higher human TBE incidence rate might also be related to an increased rate of outdoor activities in potentially tick-infested areas and the spreading of those areas [10, 15], especially in urban locations and suburbs [16, 17]. However, the prevalence of TBEV in ticks is primarily low, especially in questing ticks [18], therefore various animals were investigated as potential sentinels for TBEV detection and monitoring in suspected foci [19–21].

Small wild rodents are known as sufficient reservoir hosts of TBEV, suitable for virus amplification and maintenance, therefore they might be perfect sentinels for TBEV monitoring studies [20]. Moreover, they do not migrate long distances, tend to be populated in high numbers, and are conve-

nient to trap and collect [22]. Furthermore, rodents tend to be highly infested with ticks and are known as the main hosts of subadult stages of ticks [23, 24]. In addition, studies in highly endemic Siberia regions suggest that rodents might be persistently infected with TBEV, and most of them might carry the virus in TBEV foci [25]. Moreover, a few studies reported that TBEV-Eu might persist in wild rodents without detectable antibodies [26, 27].

Although many species of animals belonging to classes of reptiles, mammals, and birds might be TBEV-infected, clinical symptoms are mainly observed in humans and less frequently in dogs, horses, and occasionally in other accidental hosts [28]. Based on studies made in the previous century, TBE in dogs was reported rarely and it was thought that clinical disease has a high risk of lethal outcome [29]. To date, TBEV is considered to be one of the most important infectious agents causing central nervous system (CNS) infections in dogs in endemic areas [30]. Although studies suggest that the major part of TBE cases in dogs are asymptomatic, acute, peracute, subacute, and chronic courses of the disease have been reported in dogs [29, 31, 32]. Moreover, TBE might be underdiagnosed in dogs because of unawareness of possible disease even in endemic regions [32–34]. Currently, the diagnosis of TBE mainly relies on TBE-related clinical symptoms and the detection of TBEV-specific IgM antibodies in blood serum and/or cerebrospinal fluid (CSF) [35, 36] however, cross-reactions with other flaviviruses are possible [37]. Moreover, TBEV RNA detection by polymerase chain reaction (PCR) might be a valuable tool for an early diagnosis [36].

The present study sought to investigate the prevalence of TBEV in wild rodents, captured in TBE foci and dogs, residing in Lithuania's second-largest city and its vicinity. Based on the data on TBEV persistence in rodents, it was hypothesized that cultivating rodent tissue samples in murine neuroblastoma Neuro-2a cell line could increase the viral load to detectable levels. Moreover, TBEV RNA and TBEV-specific antibodies as well as clinical symptoms and risk factors for the severe course of the disease and mortality in dogs in Lithuania were investigated for the first time.

Scientific novelty

The prevalence of tick-borne encephalitis (TBE) virus in small wild rodents and dogs in Lithuania was thoroughly investigated for the first time. The present study revealed an unprecedentedly high prevalence rate of TBEV in wild rodents, captured in TBEV foci in Lithuania. However, the detected viral load was low in the majority of samples. The results show that rodents are perfect sentinel animals for TBEV monitoring, and various factors

potentially leading to such a high prevalence are discussed here. Present work reports TBEV RNA detection in fetuses of *Apodemus flavicollis*, *Clethrionomys glareolus,* and *Microtus arvalis* for the first time. Moreover, it includes the first report of natural TBEV infection in *Mus musculus* mice.

The prevalence rate of TBEV RNA and TBEV-specific antibodies in dogs was investigated, and the possible risk factors for severe course of disease and death were analyzed for the first time in Lithuania. Such studies of the prevalence of TBE in dogs are very scarce worldwide and the data reported here is of high scientific novelty, raising awareness of clinical TBE in dogs. In addition, the results provide data on TBEV RNA detection in early and acute disease.

For the first time, the cell culture method was successfully used to investigate the prevalence rate of TBEV RNA in wild rodents. The results demonstrate that the cell culture method is highly efficient for increasing TBEV load to detectable quantities in the samples of wild rodents and ticks, detached and collected from the dogs.

Aims and objectives of the study

The study aimed to investigate the prevalence of TBEV in wild rodents, dogs, and detached ticks, collected from the dogs.

Four objectives were in turn devised to implement the aim of the thesis:

1. To investigate the prevalence of TBEV RNA and TBEV-specific antibodies in the samples of dog blood and to analyze the main risk factors for the severe course of the disease and death.
2. To investigate the prevalence of TBEV in suspensions and cell culture isolates of samples of ticks, detached and collected from dogs.
3. To investigate the prevalence of TBEV in the brain and internal organ samples of wild *Muridae* and *Cricetidae* rodents, captured in TBE foci.
4. To assess the detection sensitivity of TBEV RNA in wild rodent organ samples and ticks using the cell culture method and the standard molecular methods.

1. LITERATURE REVIEW

1.1. TBEV taxonomy and structure

Tick-borne encephalitis virus (TBEV) belongs to the genus *Orthoflavivirus* (*Flaviviridae* family), which includes multiple tick- and mosquito-transmitted human and animal viruses, such as West Nile fever virus, Dengue virus, Zika virus, and Japanese encephalitis virus [38, 39]. Most orthoflaviviruses are transmitted by mosquitos and are extensively studied due to their role in human health care. On the other hand, tick-borne flaviviruses, such as TBEV, Omsk hemorrhagic fever virus, Langat virus, Powassan virus, Kyasanur forest disease virus, and the emerging Alkhurma hemorrhagic fever virus, have gained significantly less attention compared to their mosquito-borne counterparts. Therefore, the main part of the data on the structure and replication of TBEV are based on research on other orthoflaviviruses [40].

Flaviviruses undergo a maturation process during replication inside the cells, and infected cells produce at least three types of particles: immature non-infectious particles, partially mature, and mature infectious particles [40, 41]. The mature virions of TBEV are smooth, and spherical, with a diameter of 50 nm, which is comparable with other flaviviruses [42]. TBEV is an enveloped virus with a positive-sense single-stranded RNA genome. The genome is approximately 10.5 kb and includes a 5’ type I cap, 5’- and 3’- non-coding regions, and one long open reading frame (ORF). The ORF encodes three structural proteins (C, prM, and E) and seven non-structural proteins (NS_1, NS_2A, NS_2B, NS_3, NS_4A, NS_4B, and NS_5) [40, 43]. The virion consists of a nucleocapsid, surrounded by an envelope, which is composed of host-derived lipids, in which two viral membrane proteins, the major envelope glycoprotein E (molecular mass, 52 kDa) and the small membrane protein M (molecular mass, 7–8 kDa) are embedded [44]. The lipid envelope is slightly angular due to distortion by transmembrane domains of E and M proteins, as is commonly observed among flaviviruses. In addition, the surface of the TBEV virion is covered with small protrusions formed by glycans attached to the subunits of the E protein [42]. The protein E mediates viral entry via receptor-mediated endocytosis. Moreover, it contains the major antigenic epitopes, which induce protective immune responses in the host [45].

1.2. Genetic diversity and geographical distribution of TBEV

Three main TBEV subtypes were set apart by Ecker et al. [46] according to their primary geographical distribution: European (TBEV-Eu), Siberian (TBEV-Sib), and Far-Eastern (TBEV-FE). Interestingly, the amino acid sequence variation in polyprotein is low: up to 2.2% within and up to 5.6% between TBEV subtypes [46]. However, the taxonomy of the TBEV subspecies was further complicated after the discovery of the divergent TBEV strains 178-179 and 886-84 in Eastern Siberia near Baikal in 2001, which could not be accurately assigned to already known subtypes [47]. Although the strains mentioned above form a common clade with the Far-Eastern subtype, some authors consider them two separate subtypes of TBEV [48–50]. Recently, the name of the Baikalian subtype was given to "886-84-like" viruses [50–52]. However, the taxonomic position of strain 178-79 remains debated [53]. In 2017, a highly divergent strain TBEV2871 (prospectively termed Obskaya) was reported to be found in Western Siberia in an adult *Ixodes pavlovskyi* tick collected in the Ob' riber vicinity [43]. In 2018, another new subtype was described in marmots (rodents) in the Tibetan Highlands and the Himalayan subtype name was proposed to name it [49]. At least three separate lineages have been reported within the TBEV-Sib subtype [43]. In addition, different genetic lineages have been identified within each subtype. Several TBEV-Eu lineages have been described [54–57]. In addition, a new lineage of the TBEV-Eu subtype was found in 2015 in the Netherlands, a country previously considered free of TBEV [58]. Within the TBEV-FE subtype, at least four separate lineages have been found and reported [47, 59–61]. However, the status of these novel viruses in terms of subtype names remains uncertain and therefore varies in different publications [62]. In addition, a sister clade to the European TBEV subtype is a tick-borne Louping ill virus (ovine flavivirus), endemic to many areas in Europe, from the British Isles to the Mediterranean [63].

In recent decades, multiple studies have reported the isolation of TBEV subtypes far from their nominal geographic region [64]. TBEV-Eu is carried mainly by *Ixodes ricinus* ticks in central and north-eastern Europe, whereas TBEV-Sib and TBEV-FE are mostly found in *I. persulcatus* ticks in areas from north-eastern Europe to the Russian Far East, China and Japan [65, 66]. All three TBEV subtypes have been found in the Crimean Peninsula in Ukraine [67], Latvia [68], and Estonia [69]. Unexpectedly, the TBEV-Eu subtype has been found in South Korea, while in the neighboring countries (Japan, China, and northeastern Russia) only the TBEV-FE subtype has been isolated [66, 70]. In addition, TBEV-Eu subtype has been detected in Russia's Altai and Irkutsk regions [62]. The TBEV-Sib subtype is found in Mongolia

[71], Kyrgyzstan [72], Sakhalin Island [73], parts of the Scandinavia region [74], and Bosnia, where a novel lineage of the TBEV-Sib subtype has been discovered in addition to already known lineages [75]. The TBEV-FE subtype has been detected in the Urals, Southern Siberia [62], and Moldova [76].

An increasing body of data on TBEV expanding into new areas has been reported in the past decade. TBEV expansion to higher altitudes has been reported in Czechia [77] and Italy [78]. Moreover, Soleng et al. [79] were the first to report TBEV prevalence in ticks, collected at locations up to 65.1 degrees North in Northern Norway. Furthermore, human TBEV has been reported in countries previously considered TBE-free, such as the Netherlands [80,81], and the United Kingdom [82]. Additionally, TBE incidence is increasing and new foci have emerged in France [83], Denmark [84], Northern and Southern Germany [85–87], and Sweden [88].

1.3. Vectors of TBEV and biology of *Ixodes ricinus* ticks

The main vector of TBEV-Eu is *I. ricinus* ticks while *Ixodes persulcatus* ticks are the main vector of TBEV-Sib and TBEV-FE [89]. Moreover, studies show that *Ixodes inopinatus*, which may predominate in the western Mediterranean and also was detected in Germany, Austria, and Romania [90] might have been mistaken for *I. ricinus* [91]. Topp et al. [85] reported TBEV prevalence in ticks of *Ixodes ricinus/inopinatus complex* in Germany. The distribution of *I. ricinus* overlaps with *I. persulcatus* in the east, where *I. persulcatus* is found in parts of Eastern Europe and throughout temperate Asia [92]. In TBEV-endemic areas where the species mentioned above are absent or prevalent in low numbers, other *Ixodid* ticks may act as vectors, including *Haemaphysalis concinna* ticks, an important TBEV vector in Asia [93, 94]. From the north to the south of Eurasia the forest zones transit to forest-steppe (a suitable habitat for *Ixodes* and *Dermacentor* ticks) and steppe (where *Dermacentor* ticks are dominant) [93]. Recently, TBEV was detected in *Dermacentor reticulatus* ticks in multiple studies in Poland [95–97] and Germany [98]. Ličková et al. [99] suggested that *D. reticulatus* is potentially a biologically effective vector of TBEV. In some endemic foci, it might be an underrecognized TBEV vector that contributes to the expansion of TBEV in endemic areas. An experimental study demonstrated TBEV persistence in *D. reticulatus* ticks and tick cells [100]. Other possible vectors capable of carrying and transmitting TBEV may be *I. pavlovskyi*, *Ixodes ovatus*, *Haemaphysalis flava*, *Ixodes nipponensis*, *Dermacentor nutalli,* and more [93]. However, as concluded by Labuda and Randolph [101], TBE virus survival depends not on the specific virus susceptibilities of certain vector species (as

for many other arboviruses), but rather on the intimate ecological association of *I. ricinus* or *I. persulcatus* with transmission-competent vertebrate hosts.

I. ricinus ticks spend most of their life (≥ 99%) off the host, either unfed (resting or questing), engorged (in a development diapause (physiologically-mediated delay)), or engorged and developing into the next stage (moulting) following a blood meal [102]. *I. ricinus* is a three-host tick and under favorable conditions, the life cycle usually requires 2–3 years in the south range and 4–6 years in the northern range [102, 103]. Each of the three active stages (larva, nymph, and adult female) takes just one huge blood meal throughout a few (usually 4–10) days. Freshly engorged and detached ticks search for a place to avoid direct exposure to sunlight and impending desiccation. In areas with low precipitation, permanent leaf litter is crucial for the survival of *I. ricinus* ticks [102, 104]. The adult female tick lays eggs after the last blood meal. The adult male tick usually does not feed, although it sometimes takes a small blood meal. The adult male ticks search for a host mainly not to feed, but to mate. Males are commonly found mating females when feeding bloodmeal [105]. The larva or the nymph of *I. ricinus* tick develops into the next stage within a few weeks or months after the engorgement and detachment (depending on ambient temperature) or following a diapause over the winter [103, 106]. As *I. ricinus* ticks actively quest for hosts and therefore spend most of their lifecycle off their hosts, their survival and development depend on the environmental conditions, including abiotic conditions, habitat quality, and host availability [107–109].

Host-seeking activity (questing) of nymph and adult *I. ricinus* ticks has been reported at 3 °C or slightly below. However, the approximate lower threshold of daily temperature for the activity of significant numbers of nymphs and adults is around 10 °C [108, 110]. Hvidsten et al. [111] reported a northern extension of the range of *I. ricinus* in Norway concluding that the effect of temperature on tick activity is unlikely to be the main determinant of tick population establishment. The daily temperatures in the newly detected *I. ricinus* northern range regularly exceed the tick activity temperature threshold of 7–10 °C only for a few months from June to September. However, such a short period seems sufficient to maintain the *I. ricinus* population and the development to the next stage before the onset of unfavorable conditions. Moreover, the growing season length (GSL) was associated with the presence of *I. ricinus* and reached at least 170 days in the permanent sites of *I. ricinus* [111].

Tick activity is also strongly dependent on the humidity of the environment and the body-water status of the ticks, especially at higher temperatures [108, 112]. The activity may be temporarily depressed when habitats become

too dry, even if maximum temperatures remain below the upper threshold of 35 °C. The questing activity may fluctuate considerably over only a few days, especially after rainfall when the level of questing ticks may quickly increase and reach the former high [102]. If the relative humidity is 80–85% or higher, ticks can actively absorb water from the surrounding air. On the other hand, ticks lose body water if the relative humidity falls below 80% [113] or the saturation deficit is too high [108, 114]. However, Gray [105] reported that questing of all *I. ricinus* stages can persist in hot weather, as long as the litter layer remains sufficiently moist, and the ticks can retreat into the soil and leaf layer to restore body water.

Typical *I. ricinus* habitats are forests including margins and relatively humid forest-like biotopes such as bushes and thickets, heaths, moorland, heathland, scrub and rough pasture, and unmanaged grasslands [9, 115, 116]. Woodlands and broad-leaf forests with sufficiently dense undergrowth are the most preferable, however, the ticks can also be found in appreciable densities in coniferous forests if there is a sufficient amount of precipitation [102, 115]. In the far northern *I. ricinus* range ticks are found in scrubs and coniferous forests [111] and in the southern range, ticks prefer mountainous habitats with scrub and sparse woodlands [117]. In addition, *I. ricinus* is also found in sheep-grazing pastures and humid valley pastures in the British Isles, which are not common biotopes in continental Europe [118]. Moreover, ticks are present throughout Europe in urban green areas with high relative humidity, such as parks and gardens, forest patches, and cemeteries [17, 116, 119].

Numerous studies have investigated the abundance and distribution of *I. ricinus* in forests and woodlands with reported densities ranging from 75 to 488 nymphs per 100 m^2 [120–122]. The investigation of the prevalence of *I. ricinus* ticks in cattle grazing pastures in France conducted by Boyard et al. [123] reported a density of 27 to 388 nymphs per 100 m^2. A lower abundance of *I. ricinus* nymphs in grassland compared to woodland was also reported in Scotland [120]. The abundance of ticks in pastures of Ireland was associated with the availability of tick hosts [124]. Various factors, such as humidity and the presence of woodland or bushy vegetation on the perimeter of the grazing pasture potentially influence the abundance of ticks by providing tick litter mats and sufficient moisture [125]. A study in France revealed that moder humus soil type, characterized by a thick layer of fragmented leaves was strongly associated with nymph abundance (compared to mull and mull-moder humus soil) [126]. Woodlands and hedgerows are natural shelters for small vertebrates and rodents, such as *C. glareolus* and *Apodemus* spp. [127], which are well-known hosts not only for larvae and nymphs of ticks [128, 129] but also for TBEV [130]. In addition, *Apodemus sylvaticus* can also be

found in pastures [131]. Other mammals that seek shelter in bushes and thickets are roe deer (*Capreolus capreolus*), which are important hosts of *I. ricinus* ticks [132, 133] and providers of mating places for adult ticks [105]. Interestingly, a study in France found that the apple and cherry trees on the pasture's perimeter benefit the abundance of *I. ricinus* ticks. The authors discussed that fruits attract birds and mammals, which might contribute to a significant population of ticks near the fruit trees [123].

Host-seeking *I. ricinus* ticks primarily respond to non-specific stimuli. Gradient carbon dioxide changes, increase in local temperature, presence of a sudden shadow, and vibration attract *I. ricinus* ticks [110]. There is some evidence that *I. ricinus* are attracted to the volatile substances from the back of a dog's ear [134], eructations of volatile rumen metabolites of ungulates [135], and the odor of rodents (bank voles) [136]. Moreover, substances from deer tarsal glands and dorsum of the dog ear induce a response of ticks (*Ixodes scapularis*, *Amblyomma americanum*, *Dermacentor variabilis*) to aggregate in places associated with host presence [137].

1.4. TBEV maintenance in nature

According to the World Health Organization, arboviruses are "viruses that are maintained in nature principally, or to an important extent, through biological transmission between susceptible vertebrate hosts by hematophagous arthropods or through transovarial and possibly venereal transmission in arthropods: the viruses multiply and produce viremia in the vertebrates, multiply in the tissues of arthropods, and are passed on to new vertebrates by the bites of arthropods after a period of extrinsic incubation" [138]. The principal transmission route of tick-borne viruses is horizontal, from an infected tick to an uninfected definitive host and from an infected host to an uninfected tick [139]. According to Nuttall and Labuda [140], horizontal TBEV transmission between ticks and their vertebrate hosts is necessary for virus endemism. Generally, the hosts are divided into three groups: reservoir, indicator, and accidental. Natural reservoir hosts of TBEV (animals important for the transmission of TBEV to ticks since they are sensitive to the virus, and show prolonged viremia without the presence of clinical illness) are rodents (*Apodemus*, *Clethrionomys*, *Microtus*, *Mus*, *Micromys*, *Pitymys*, *Glis*, *Arvicola*, *Sciurus,* and *Citellus*), insectivores (*Erinaceus*, *Sorex*, *Talpa*) [141] and carnivores (*Vulpes*, *Mustela*) [27, 142].

Although horizontal transmission from vertebrate to tick has been previously suggested to depend on the level of viremia, it is now known that tick-borne encephalitis virus can be transmitted during the co-feeding of

infected and uninfected ticks on non-viremic hosts [143, 144]. A recent experimental study demonstrated that TBEV viremia in bank voles (*C. glareolus*) may last up to 28 days, although the possible virus infectivity for ticks was not tested [145]. To date, transmission during co-feeding between infected nymphs and uninfected larvae when they feed near each other on the same host is thought to be the main transmission route of TBEV-Eu and the most efficient mechanism to maintain TBEV-Eu in a given area [146, 147]. Moreover, non-viremic transmission between TBEV-infected and non-infected ticks during co-feeding on the same host can be replicated empirically *via* needle-and-syringe inoculation if tick saliva or salivary gland extracts are included in the virus inoculum [148]. The pool of saliva produced by ticks that feed near each other is thought to enhance the virus exchange between the co-feeding ticks [146]. Enhancement of pathogen transmission by ticks' saliva, called saliva-assisted/activated transmission has been documented for several tick-pathogen relations [149]. Furthermore, a TBEV transmission during co-feeding can also occur in previously TBEV-immuned hosts, although the effectiveness is reduced compared to co-feeding on non-immuned hosts [101].

Vertical transovarial transmission is another possible mode for ticks to transmit TBEV. However, various studies report that only 0.23–0.75% of larvae hatched from eggs laid by TBEV-infected adult females were transovarially infected and TBEV-positive [150, 151]. Other studies report that the prevalence of transovarial TBEV transmission is generally < 5% [152]. The rate and prevalence of transovarial TBEV transmission are probably insufficient to maintain TBEV in nature without the amplifying effect of horizontal transmission [153]. However, it is considered as important since larvae show a highly non-random distribution on their host, and usually, the ticks from an egg batch quest together, therefore, the infection rate might be enhanced as a result of non-viremic transmission among co-feeding larvae [154]. Moreover, trans-stadial TBEV transmission occurs meaning that once infected, the tick will usually remain infected throughout its life [140]. Furthermore, sexual transmission when TBEV-infected tick males infect females by transferring infectious saliva or seminal fluid during copulation is another mode of virus transmission between ticks [155]. However, it is not clear if transovarial and sexual transmission of TBEV are required for the long-term persistence of TBEV in the ecosystem [156]. In addition, male ticks (*I. ricinus* and *I. persulcatus*) sometimes feed on hungry females off the host, therefore so-called "omovampiric" transmission of TBE by males of *I. persulcatus* is possible according to Alekseev [155]. This theory was raised because there were enough infective virions in the male tick saliva and a high incidence of "omovampirism" (> 7% of field-collected females had male bite traces) [155].

1.5. Hosts of TBEV

1.5.1. Wild animals

The larger hosts (e.g., cervids, foxes) tend to carry higher quantities of ticks, although they are usually found in much lower densities than rodents. Randolph et al. concluded that the abundance of rodents, even if the intensity of tick infestation is smaller, certainly compensates and rodents might be hosting the major part of the local tick population at any given time [103]. Moreover, rodents in temperate climates, such as Central Europe, show cyclic population fluctuations with the lowest population numbers in early spring and the highest in late summer/early autumn [157]. This peak of rodent abundance in early autumn overlaps with the density peak of *I. ricinus*, especially nymphs. In addition, in early spring when rodents tend to be less abundant, the densities of adult ticks are highest, which prefer a larger host [104].

Rodents of various species were reported as hosts for ticks in Central Europe [158]. However, several studies have suggested that some species of rodents might be more preferred hosts of larvae and nymphs of *I. ricinus* ticks than others. A study conducted in Germany reported *A. flavicollis*, *Apodemus agrarius*, and *C. glareolus* [159] in France [160] and Romania [161] *M. arvalis*, *Apodemus uralensis,* and *A. sylvaticus* rodents as main hosts for subadult stages of *I. ricinus* ticks. Although rodents are highly competent hosts for ticks, especially for subadult stages, post-larval bite-acquired resistance to *I. ricinus* ticks was reported in *C. glareolus* voles, which results in reduced engorged weight and reduced survival of nymphs. However, the same study did not observe resistance to *I. ricinus* in *A. flavicollis* mice [162]. Resistance to *Ixodes trianguliceps* ticks in *A. sylvaticus* was much lower compared to laboratory mice [163]. In addition, individuals of *C. glareolus* and *A. sylvaticus* with high testosterone levels showed reduced innate and acquired resistance to feeding *I. ricinus* ticks [164]. Several studies reported lower tick burdens in *C. glareolus* compared to *Apodemus* spp. [160, 165–168]. A better roaming and a greater home range occupied by *Apodemus* spp. are suggested as potentially influential for higher tick burdens on mice [165]. Although Kiffner et al. [169] have not found a significant difference in tick burdens on *C. glareolus* and *A. sylvaticus*, Boyard et al. [170] reported that *C. glareolus* had significantly lower quantities of ticks than *Apodemus* spp. and even slightly lower than in *M. arvalis*, which are pasture-specific. Moreover, tick burden was influenced by the rodents' habitat: *A. sylvaticus* carried 4.8-fold more larvae in woodland and 2.6-fold more larvae in hedgerows compared to grassland not connected to woodland [170].

Deer, especially roe deer (*Capreolus capreolus*) and red reed (*Cervus elaphus*), are native animals in northern and central Europe and are natural hosts for *I. ricinus* [102]. Several studies have observed a clear association between deer density and *I. ricinus* abundance [171–176]. However, Hofmeester et al. [177] suggested that the presence of deer, rather than density is sufficient to ensure high tick abundance. Nonetheless, Dobson and Randolph [178] demonstrated the complexity of the deer density and tick abundance relationship by mathematical models suggesting that the density effects varied with the initial density of deer.

Cervids are known to be important hosts for adult tick feeding and mating, although multiple studies suggest that deer might also be important hosts for subadult stages of ticks. Cervids, especially roe deer might carry significant numbers of subadult tick stages, which might not be noticed because of the specifics of the methods of tick counting on carcasses of large animals [179]. *I. ricinus* nymphs (50.5%) were reported as the most frequent tick stage found on red deer, followed by adult females (34.8%), males (11.4%), and larvae (3.3%) [180]. Another study reported that nymphs (23.9±3.2) were slightly more abundant compared to adult females (21.4±3.5), larvae (10.8± 4.2), and males (8.4±1.5) [181]. On the other hand, Król and colleagues [182] described that *I. ricinus* was the predominant tick species (99.76%) found on the red deer and adult females were the dominant life stage (42%), followed by nymphs (31.1%), males (13.7%) and larvae (13.2%). Moreover, an almost five-fold higher mean number of ticks found on the red deer in the forest ecotype (3.75±0.83) compared to the field ecotype (0.77±0.20) was revealed and suggested that the habitat of deer might play a role in the density of tick infestation [183]. Furthermore, an increase in roe deer abundance due to the changes in land, vegetation, and wildlife management practices, that support rodent populations, is likely to be among the most crucial factors affecting the circulation potential of TBEV-Eu [184].

The role of cervids in the transmission cycle of TBEV is mostly limited to incompetent transport hosts, which can distribute ticks across their foraging range with enclosure areas of 30 km^2 or larger [180, 185]. A spatial analysis study by Knap and Avšič-Županc [186] found a positive correlation between red deer and roe deer abundance and TBE incidence at the country level in Slovenia. At the local level, a correlation between red deer abundance and tick-borne encephalitis occurrence was confirmed, however, no such relations were noticed between roe deer density and TBE incidence in Slovenia [186]. Kriz et al. [187] concluded that the regulated population size of roe deer seems not to be implicated in recent geographical and temporal variations in TBE incidence in Czechia. Moreover, a “dilution effect hypothesis” was raised by Cagnacci and colleagues [188] who observed that TBE infection

in ticks and rodents was positively associated with the number of co-feeding ticks and negatively correlated with deer density in Italy and Slovakia. The authors hypothesized that the negative relationship between deer density and TBE incidence on a local scale could be attributed to deer (incompetent hosts) diverting questing ticks from rodents (competent hosts) [188]. Perkins et al. [189] proposed that the localized absence of deer (loss of dilution host) increases tick feeding on rodents, leading to the potential for tick-borne infection hotspots.

The seroprevalence of TBEV-specific antibodies in cervids varies between countries and surprisingly high seroprevalence was reported in countries of low TBEV endemicity such as Belgium and Italy. The reported TBEV-seroprevalence in roe deer was 0.7% [190] and 19.5% (1.6% and 40.7% in different areas) in Norway [191], 2.4% (4.1% including borderline results) in Austria [192], 2.5% in Serbia [193], 4% in England and Scotland [82], 6.9% [194] and 8.7% in Denmark [195], 11.6% in Poland [196], 12.4% in Belgium [197], 37.7% in Italy [198], 50–53% in Sweden [133, 199]. Interestingly, highly variable results were reported by a few separate studies in different areas of Germany, with reported seroprevalence of 2.7% [200], 7.8% in Northeastern Germany [201], 22.4% in Southern Germany [132], and 22.9% (varied between 17.6% and 50% in two forest districts located in the same county) in separate studies [203]. Moreover, TBEV seroprevalence in other cervids was reported to reach 42% in moose (*Alces alces*), 42% in red deer and 25% in fallow deer (*Dama dama*) in Sweden [199], 22.6% in red deer and one out of eight tested chamois (*Rupicara rupicara*) in Italy [198], 1.7% in red deer and 5.6% in fallow deer in Germany [200], 1.4% in red deer and 9.4% in moose in Norway [190], 0.74% in moose and 0.74% in white-tailed deer (*Odocoileus virginianus*) in Finland [204], 0.5–2.4% in sika deer (*Cervus nippon*) in Japan [205]. TBEV seroprevalence of 63.5% in Poland was reported in European bison (*Bison bonasus*) [206]. Furthermore, TBEV RNA was detected in 1.1% of spleen samples of red deer, hunted and collected in Croatia [207]. In addition, a clinical TBE case in roe deer was reported in Italy [208].

The role of wild boar (*Sus scrofa*) in TBEV maintenance is unclear. Kriz et al. [187] suggested that the size of the wild boar population may have contributed to the current high and rising trend in the incidence of TBE in Czechia. The seroprevalence of 32% was reported in Sweden [199], 17.4% in Italy [198], 16.8% in Poland [196], 2.9% (or 4.2% including borderline results) [209], and 9.3% (7.7–33.4% in different areas) in Belgium [210], varying from 0% to 23.3% in various locations in Germany [200, 202], 7% in the Netherlands [211], 12.5% in Serbia [193] and 0.9% in France [212].

Red foxes (*Vulpes vulpes*) are important predators of young deer, and more importantly – a key predator of small mammals, such as rodents. The

red foxes might play a significant role in the reduction of tick quantities and as a consequence, the risk of tick-borne infections [15]. Reported tick infestation of red foxes in Europe ranges from 7.4% in Italy [213], 51.1% [214] to 82.6% in Germany [215], and 86% in Hungary (45% of collected ticks were *I. ricinus*, although the intensity of infestation was low (5.2±0.9)) [216]. Takumi et al. [217] suggested that the abundance of foxes and leporids negatively affected tick abundance in the Netherlands. Kiffner et al. [218] found that the hunting bag density of red foxes in the previous year was negatively correlated with human TBE incidence in Germany. Contrarily, a positive correlation between the number of human TBE cases and the number of red foxes was found in Sweden [219]. Higher quantities of red foxes were associated with a higher incidence of human TBE cases in the following year. However, the densities of European hares (*Lepus europaeus*) and grouse (*Tetraonini*) negatively correlated with human TBE incidence in the same study in Sweden [219]. Although the studies of TBEV in red foxes are scarce, a few conducted in Germany showed that red foxes are promising sentinel animals for TBEV monitoring. The investigation of TBEV-specific antibodies in red foxes conducted in Germany by Haut et al. [220] revealed a seroprevalence of 30.5% in TBE risk areas and a significantly lower seroprevalence of 13.1% in non-risk areas (overall 20.1%). In various locations in Germany, TBEV-seroprevalence of 1.8–34.2% was found in red foxes (results not confirmed by VNT) [221]. In non-endemic areas in Germany, 1 out of 759 red foxes was TBEV seropositive in a study conducted almost three decades ago [222]. In an investigation in the Netherlands, a seroprevalence of 0.5% was detected in the blood of red foxes (results not confirmed by VNT) [211].

1.5.2. Domestic animals

Sheep were originally introduced from the Middle East and are not natural hosts for *I. ricinus* ticks (despite the common English name of sheep tick for *I. ricinus*) [223]. Although sheep and domestic ungulates can maintain *I. ricinus* populations when other hosts are absent [224], sheep can develop strong resistance to *I. ricinus* [225]. However, Dobson and Randolph [178] suggested that adding large hosts (sheep) would effectively reduce the number of questing ticks and therefore the risk to humans. Moreover, if the sheep are treated with acaricide, the populations of ticks are predicted to decrease rapidly. The extent depends on the relative abundance of untreated wild hosts and treated domesticated hosts in a given area [178]. On the other hand, studies in forest habitats grazed by sheep or cattle observed a decrease

in tick densities which might be explained by the reduction of vegetation litter after grazing [226, 227].

The prevalence of TBEV-specific antibodies in the blood serum of sheep in Europe suggests that sheep are efficient hosts for ticks and TBEV. The prevalence of TBEV-specific antibodies in sheep in Germany varied from 0% to 12.8% in different regions [228]. The TBEV-seroprevalence reached 7.7% in Sweden (25.6% in farms with high lamb morbidity and mortality) [229], 7% in Hungary [230], 4.2% in Lithuania [231], 2.6% in Slovakia [232], and 0.4% in Belgium [210].

The reported prevalence of TBEV-specific antibodies in the blood serum of goats in Europe varied. The highest seroprevalence reaching 14.6% was revealed in Switzerland [233]. In addition, a seroprevalence of 4.2% was reported in goats older than 6 months in another study in Switzerland [234]. A TBEV-seroprevalence ranging from 0.4% to 13% was reported in Germany [228, 235]. A seroprevalence of 4.7% was reported in Poland [236], 0.9% in Austria [237], and 0.7% in Lithuania [231]. An alimentary outbreak in Croatia was confirmed following the detection of TBEV-specific antibodies in the blood serum of goats on the suspected farm [238]. Moreover, a new TBEV focus was revealed in Italy by screening goat blood serum for TBEV-specific antibodies [239].

The reported TBEV-seroprevalence in cattle varied in different countries in Europe. The highest rate of TBEV-specific antibodies was reported in Hungary, reaching 26% [230]. Paulsen et al. [240] reported a seroprevalence of 13.4% in blood samples of cows. TBEV-specific antibodies were detected in 4.1% of cattle blood serum samples in Poland [196], 2.6% in Belgium [241], and 2.4% in Lithuania [231].

A few studies have investigated the prevalence of TBEV RNA and TBEV-specific antibodies in the milk samples of goats, sheep, and cows. A study conducted in Sweden revealed the prevalence of TBEV-specific antibodies in 5.4% of unpasteurized cow milk samples [240]. Another study conducted in Sweden reported the presence of TBEV-specific antibodies in 2.9% of cow milk samples [242]. A study conducted in Poland reported a TBEV RNA prevalence in the unpasteurized milk of 22.2% of sheep, 20.7% of goats, and 11.1% of milk samples of cows. Moreover, TBEV-specific antibodies were found in the milk of 14.8% of sheep, 3.2% of cows, and 0% of goat milk samples [243]. TBEV RNA prevalence of 4.5% in raw sheep milk and 4.3% in raw goat milk was reported in Lithuania [244].

The highest reported prevalence rate of TBEV-specific antibodies in horses was in Lithuania, reaching 37.5% [245]. A high seroprevalence of 26.1% in horses was reported in Austria [246]. Various prevalence rates of TBE-specific antibodies in horses were reported in Germany in a few studies,

fluctuating from 0.8% in non-endemic areas and 20-33% in TBEV-risk areas [235, 247–250]. The reported prevalence rate of TBEV-specific antibodies in horses was 3.4% in Slovakia [251] and 3.1% in Spain [252]. Moreover, clinical cases with severe neurological symptoms were reported in three horses in Austria [253, 254], and seven horses in Switzerland [255, 256].

To date, no reports of the presence of TBEV-specific antibodies in the blood serum of cats are available. Blood serum samples of 399 cats (89% of them had outdoor access) residing in Germany were analyzed for TBEV-specific antibodies. No positive results were found among cats. However, one sample showed a borderline result, which was negative in the serum neutralization test (SNT) [200]. Two other studies have investigated blood serum samples of 221 and 143 cats in Germany and did not detect TBEV-specific antibodies [257, 258]. Whether cats mount an antibody response after contact with TBEV remains unanswered [200].

The data on the prevalence of TBEV-specific antibodies in dogs are reviewed in Section 3.1.1.

1.5.3. Birds

In Europe, passerine birds are hosts of immature *I. ricinus* ticks, the vectors of a wide range of zoonotic pathogens, including TBEV. As a result, birds can transport and disseminate infected ticks along their migration routes [259]. Most migratory birds divide their trip into several so-called migration legs, interspersed with time spent on stopover sites along their migration route and providing opportunities for new ticks to attach and already-fed ticks to detach from the birds [260]. Following birds along their migratory routes, the TBEV-infected ticks may allow the emergence of new endemic foci, only if biotic and abiotic conditions are favorable for the maintenance of the virus in ticks and vertebrate hosts [259].

Migratory birds have been found to harbor TBEV-infected ticks in various European countries. TBEV RNA prevalence of 14% in *Ixodes* ticks, collected from birds of different species in Latvia [261]. In Estonia, TBEV-Eu RNA was found in one *I. ricinus* nymph removed from Marsh Warbler (*Acrocephalus palustris*) [262]. TBEV was found in two single nymphs (0.7%) collected from two different Common Blackbirds (*Turdus merula*) in Kaliningrad Oblast, a Baltic region of Russia [263]. Six TBEV-positive ticks were found attached to birds (one larva on a juvenile Tree Pipit (*Anthus trivialis*), one nymph on a juvenile Common Redstart (*Phoenicurus phoenicurus*), two nymphs and one larva on a juvenile European Robin (*Erithacus rubecula*), and one nymph on Song Thrush (*Turdus philomelos*) during autumn migration in Sweden [264]. In Switzerland, birds have been suspected to play

a role in the emergence of new TBE foci [56, 265], and TBEV was sporadically found in ticks collected from birds in Switzerland [259].

A study conducted in the region of Western Siberia tested samples of birds and ticks, collected from birds for the markers of TBEV using monoclonal modified enzyme immunoassay (EIA) and RT-PCR. TBEV RNA was detected in 9.7% and TBEV antigen in 22.8% of wild bird samples. Moreover, TBEV markers were also found in 14.1% *I. persulcatus*, 5.2% *I. pavlovskyi*, and 4.2% *I. plumbeus* ticks, collected from the wild birds [21]. Korobicyn et al. [266] concluded that the emergence of TBEV-FE in *I. pavlovskyi* ticks in the Western Siberia region was directly caused by birds. Moreover, TBE viral RNA and viral antigen were detected in the internal organs of 7.3% of migratory birds (such as Brambling (*Fringilla montifringilla*), Common Redstart (*Phoenicurus phoenicurus*), Fieldfare (*Turdus pilaris*), Chiffchaff (*Phylloscopus collybita*), Great Tit (*Paurus major*), European Pied Flycatcher (*Ficedula hypoleuca*), Lesser Whitethroat (*Curruca curruca*), Redwing (*Turdus iliacus*), Common Magpie (*Pica pica*), Hooded Crow (*Corvus cornix*), Common Starling (*Sturnus vulgaris*), Rock Pigeon (*Columba livia*), etc.) Furthermore, the authors reported that the prevalence of TBEV markers in birds frequently infected by ticks did not differ from that in birds with rare contact with ticks. In addition, the number of detected pathogens in individual birds did not correlate with the number of ticks, parasitizing them [266].

The reported data suggests that birds can potentially spread TBEV in new areas. The reported TBEV presence in wild birds and ticks, parasitizing them indicates that birds may also play a role in the virus maintenance in nature. Although more detailed research is needed, the existing data show that viral RNA detection in the hosts and vectors of TBEV is inefficient in evaluating the true prevalence of TBEV.

1.6. Transmission routes and clinical symptoms of TBE

1.6.1. TBE in humans

The main transmission route for TBEV is via the bite of an infected tick [1]. The most common non-vectorial TBEV transmission route is following the consumption of unpasteurized milk and milk products from infected goats, sheep, and cows [267, 268]. Other transmission routes, such as materno-fetal [269] and transmission through breastfeeding [238] were recently reported. TBEV transmission through organ transplant was reported in Poland not long ago, where three patients who received solid organ transplants from a single donor developed encephalitis 17–49 days after transplantation and subsequently died [270]. In addition, TBEV transmission through blood transfu-

sion was reported in two patients in Finland more than 30 years ago [271]. Transmission through handling TBEV-infected materials in the laboratory was reported in three patients in the abovementioned study in Finland [271] two patients in Germany more than 40 years ago [272] and 1 patient in Slovenia potentially infected through aerosol during isolation of the virus from infected blood [273].

Clinical TBE can be a severe disease that often results in lifelong neurological sequelae and also can lead to death. The morbidity and mortality of TBE vary depending on the viral subtype, the age of patients, and host genetic factors. The estimated mortality rate of patients infected with TBEV-Eu is 0.5–2% [4, 274]. Up to 10% of TBEV-Eu patients show neurological sequelae and up to 50% of patients after acute TBE [275]. TBEV-Sib-infected patients are prone to develop prolonged infections with a mortality rate of 2–3%. TBEV-FE is associated with high rates of neurological sequelae and up to 20–40% case fatality rate [4, 274, 276].

Various studies suggest that about two-thirds of human TBE infections are asymptomatic [4, 277, 278]. Moreover, about one-third of TBE human patients do not notice or recall the tick bite. The incubation period of TBE ranges from 2 to 28 days and on average is 7 to 14 days after the bite of an infected tick (shorter after alimentary infection). The length of the incubation period and the severity of the subsequent illness do not correlate [275, 277]. Men are affected twice as frequently as women, and some studies suggest that man (especially older) has an increased risk of severe disease [279]. A biphasic course of the disease with the prodromal period is characteristic of the TBEV-Eu subtype and occurs in about 75% of patients. The initial viremic phase lasts 2 to 7 days and manifests with fever (37.5–39.0 °C), headache, muscle pain, and fatigue. An afebrile period, that lasts 2 to 10 days usually follows the first phase of TBE. Thrombocytopenia, leukopenia, and elevated liver enzymes are common hematological findings in the first phase of the disease. The fever returns (1–2 °C higher than the peak temperature in the first phase) accompanied by the symptoms of inflammation of the CNS in the second phase of TBE [4, 274, 275, 277, 278, 280].

Meningeal symptoms are present in the majority (about 50%) of symptomatic TBE cases in human beings, typically manifesting as high fever, headache, nausea, vomiting, and nuchal rigidity, accompanied by photophobia and vertigo in many patients [275]. The most typical clinical sign of TBE is ataxia of the limbs. Meningoencephalitis (seen in about 40% of TBE patients) can be displayed by numerous neurological symptoms and signs. The symptoms of meningoencephalitis caused by TBE include restlessness, hyperkinesia of face and limb muscles, lingual tremors and speech disorders,

tremors of extremities, movement disorders, pareses, convulsions, vertigo, and seizures. Mental disorder symptoms, such as impaired cognitive function and concentration, impaired consciousness (from sleepiness, amnesia to stupor, or even coma in rare cases), personality and behavioral changes (denying food, increased aggressiveness, skittishness, or apathy) delirium or psychosis may also rapidly develop in occasional cases [4, 274, 275].

Meningoencephalomyelitis (occurs in 5–15% of patients) is the most severe form of the TBE, usually associated with meningoencephalitis. It manifests as flaccid paralysis when motoneurons, radices, or peripheral nerves are involved (poliomyelitic or polyradiculoneuritic forms that affect the upper extremities more frequently than the lower ones and proximal segments more often than the distal ones) or with spastic pareses in rare cases. Severe pain in the arms, legs, and back is occasionally reported as the preceding symptom in the progress of paresis [275]. Myelitis is usually accompanied by encephalitis. In rare cases, myelitis is the only manifestation of TBE [281]. However, peripheral and autonomic nervous system dysfunction was rarely reported in TBEV and other flaviviral infections [277, 282–284]. A retrospective study of TBE patients conducted by Kleiter and colleagues [284] in Germany reported TBE associations with autonomic dysfunction including reduced heart rate variability and tachycardia, symptoms of upper and lower gastrointestinal tract dysfunction, orthostatic hypotension, and urinary retention. Moreover, infection of anterior horn neurons is a well-recognized feature of TBE, and the reduction in amplitudes of motor and sensory nerve conduction velocity (NCV) suggests involvement distally to the sensory ganglia, that is, a peripheral nervous system [282, 285]. Similarly, radiculitis occurs and sensory nerves are occasionally affected in other flaviviral infections [286, 287].

In addition, some rare clinical manifestations of TBE outside CNS have been reported. Abnormal liver function tests were reported in 22.2% of TBE patients in the initial phase of the disease in Slovenia [288] and 22% in Croatia [289]. Moreover, transient pancreatitis and myocarditis have also been occasionally reported as clinical manifestations, usually in the initial stage of the disease, but also as an individually separate clinical symptom [289]. Furthermore, TBE may be complicated by polio-like syndrome [290, 291]. A chronic form of TBEV that may manifest as *epilepsia partialis continua* has been described in patients infected with TBEV-Sib and TBEV-FE. However, a chronic (progressive) course of the disease was reported in two patients in Lithuania [280], where to date only the TBEV-Eu subtype has been detected [292].

Post-encephalitic syndrome (PES) has been reported after an acute TBE caused by TBEV-Eu in 35–58% of patients. Neuropsychiatric complaints,

including hearing and vision disturbances, cognitive deficits and depression, headaches, imbalance, and flaccid paresis or paralysis [4, 278]. The long-term neurological sequelae of TBE include tiredness, anxiety, reduced stress tolerance, nausea, dizziness, headache, insomnia, disturbances of memory or concentration, disturbances of hearing, tremors, pain in the extremities, and anomalies in electroencephalography, cranial nerve paresis, hemiparesis, paraparesis, impaired balance, and coordination [274, 293]. However, findings are difficult to interpret because of unclear distinctions between PES, other sequelae of TBE, and symptoms present in general populations, as most studies did not comprise a control group [4].

Risk factors for the unfavorable outcome of the TBE disease have been identified and reported in several studies. Older age has been associated with severe clinical course [278, 280, 294, 295]. A clear tendency for a more severe course of TBE above the age of 7 has been reported [275, 296], although severe cases have been reported even in young children and infants under 1 year of age [279]. Two studies suggest that older age and male gender are associated with a more severe course of TBE infection [297, 298]. However, in the retrospective study conducted in Lithuania by Radzišauskienė et al. [299] older age and monophasic disease were associated with a more severe disease course, but not the male gender. However, there are no available data on the possible reasons for the higher risk of severe TBE in males compared to females. A higher risk of a more severe course of infection with other viruses (including flaviviruses) in males could be associated with the androgen-induced pathogenesis of viral disease [300] and the protective effects of estrogens in females [301]. In addition, higher seroprevalence in males in multiple studies [302–305] was associated with a higher risk of exposure to ticks. The specific risk factors of TBEV include living in an endemic area or near forests, spending time in forests (working, hunting, fishing, hiking, and picking mushrooms or berries), and consuming raw milk [1, 18, 306].

An encephalitic form of acute TBE [280], focal forms of acute TBE [307], and the presence of PES have been associated with an increased risk of death [308]. Multiple risk factors, such as ataxia, pareses, need for assisted ventilation, impaired consciousness (Glasgow coma scale (GCS) < 7), abnormal magnetic resonance imaging (MRI) findings, pleocytosis $> 300 \times 10^6$ L^{-1}, CSF protein concentration > 600 mg/dL were identified in the acute phase of TBE [277]. Moreover, double infection (TBE and Lyme neuroborreliosis) was reported as a risk factor for severe TBE [309, 310]. However, Logina et al. [311] did not observe any evidence that co-infection of TBEV and *Borrelia burgdorferi* sensu lato complex produces unusual clinical manifestations, unpredicted interactions between the two pathogens, or the risk of an

unfavorable outcome of the diseases. The main reported causes of TBE mortality are diffuse brain edema and involvement of the medulla oblongata and the central portions of the brain [312].

1.6.2. TBE in domestic animals

The main route of TBEV infection in dogs is through the bite of an infected tick [313]. Alimentary transmission of TBEV following the consumption of raw TBEV-infected milk is also possible and can lead to severe disease in dogs, although less frequently [267, 314]. Severe clinical TBE and long-term sequelae of ataxia were reported in a Rottweiler of 8 years of age whose diet included raw goat's milk [314]. Recently, intrauterine vertical transmission of TBEV from the dam to the offspring was reported. All three Dalmatian puppies were 2–3 weeks of age when severe neurological symptoms developed (one had epileptic focal seizures around the eyes and ears and the other two showed generalized seizures) which led to the euthanasia of all three offspring [315].

Based on a more than 10-year-old review of a few reported TBE cases in dogs, the clinical manifestation of TBE was thought to be rare and mostly fatal [29]. The incubation period may be similar to that in humans (1–2 weeks), although no data is available in dogs. An experimental TBEV infection of three-month-old Beagle puppies caused no clinical signs or changes in body temperature. In addition, only two of four infected puppies developed low titer viremia 1–3 days post-infection (d.p.i.) [313]. According to Leschnik et al. [31], the TBEV-specific antibodies after natural infection are detectable in the blood serum of dogs for at least nine months. Four different courses of TBEV infection in dogs have been reported: peracute, acute, subacute, and chronic [29, 31, 32]. The peracute course of TBE leads to death within three to seven days and in some cases, even TBEV-specific antibodies are not detectable. The prognosis of the disease becomes remarkably better when the clinically affected dog survives the first week. The clinical signs of acute TBE tend to improve in one to three weeks after onset, often without any sequelae. A chronic course may lead to neurological signs lasting within one to six months [31].

The most commonly reported symptoms of the first phase of TBE are hyperthermia, apathy, and anorexia [30]. The most common neurological symptoms in dogs with TBE reported by Kleeb et al. [30] were ataxia/vestibular signs (51.8%), cervical pain (38.8%), seizures (31%), plegia/paresis of one or more limbs (27.7%), cranial nerve deficits (29.6%), proprioceptive ataxia (29%), cervical weakness (20%). Different most common neurological symptoms in dogs were reported in a study by Kirtz et al. [316] where 54%

of dogs showed altered consciousness and behavior, 42% had a proprioceptive deficit, 37% had motor neuron deficit, 21% showed hyperalgesia of the neck, 20% showed head tilt, facial paresis, nystagmus, hypoesthesia of the head area, vestibular strabismus and dysphagia and 12% had seizures. Other reported symptoms in dogs with clinical TBE include tremors, recumbency, opisthotonus, anisocoria, miosis, cramp of front legs, strabismus, aggressiveness, head shaking, polypnea, diffuse pain, weakness, loss of eyelid closing reflex and optic neuritis [29, 34, 317, 318].

Clinical TBE in horses has been recently reported in a few European studies. Most clinical cases reported lethargy and ataxia of sudden or acute onset [253, 255, 256]. Behavioral changes, such as anxiety, aggressiveness, severe agitation, obtundation, episodes of impulsive walking, and head pressing were reported in horses with clinical TBE [253–255]. Other reported symptoms include proprioceptive deficits, head tilt, skin hyperesthesia, reduced pupillary reflexes, reduced tone of the tongue, facial nerve paralysis, and paralysis of neck and shoulder muscles [255]. In addition, reduced consciousness, loss of balance, inability to induce motion, recumbency [253], presumed blindness, and focal and generalized seizures were reported in horses with TBE [254].

Clinical symptoms of TBE in other animals were reported only sporadically [28].

2. MATERIALS AND METHODS

2.1. Sample collection

2.1.1. Dog samples and clinical data

Clinical samples of dogs were collected from two veterinary clinics in Kaunas (central part of Lithuania) that agreed to participate in the present study. Blood and CSF samples of dogs were taken in the clinics for diagnostic purposes, no additional blood was drawn for the virus analysis. Before conducting the investigation, explicit consent was obtained from the owners of the dogs, granting authorization for the utilization of clinical samples.

Blood (n = 473) and CSF (n = 3) samples from dogs who were presented to clinics with various diseases or for prophylactic testing were randomly collected from veterinary clinics between May 2020 and December 2021. In addition, three samples of cerebrospinal fluid (CSF) from dogs with neurological symptoms were collected. The collected samples were stored at −80 °C for further analysis.

Concurrent other tick-borne diseases were diagnosed at veterinary clinics. Infection with *Babesia* spp. was confirmed after the detection of specific protozoa in erythrocytes using a blood smear. Infection with *Anaplasma phagocytophilum* was confirmed by detecting specific morulae in neutrophils using a blood smear. In addition, Anti-*A. phagocytophilum* antibodies were detected by chromatographic immunoassay test (Anigen Rapid CaniV-4 Test Kit (BioNote, INC. Hwaseong, Republic of Korea).

Data on *anamnesis vitae* and *anamnesis morbi* of the dogs, including the outcome of disease followed up for six months and was collected retrospectively (Supplementary Material Table S3). Moreover, risk factors, that potentially were associated with the severity of clinical neurological symptoms and worse outcomes of the TBE disease of viremic dogs were analyzed.

2.1.2. Tick samples

A total number of 117 attached ticks were collected when they were found on the bodies of dogs at the same visit at the veterinary clinic when blood serum samples were collected. The species of ticks were recognized under a light microscope (Leica Microsystems, Heerbrugg, Switzerland) using a previously reported taxonomic key [319]. Collected ticks were determined as 112 *I. ricinus* and 5 *D. reticulatus*. Collected ticks were stored at −80 °C for further analysis.

2.1.3. Small wild rodent samples and sampling sites

Wild rodents were trapped in Lithuania in 19 TBEV foci (in 5 districts) (Supplementary Material Table S2). Specific sites for rodent trapping were selected based on the previously collected data on TBEV prevalence in Lithuania from two different sources. The exact sites of rodent collection were chosen according to a complete map of confirmed human TBE cases with probable TBEV-infected tick bite locations or known TBEV foci from 2016 to 2018 provided by the National Public Health Center under the Ministry of Health (*https://nvsc.lrv.lt/lt/uzkreciamuju-ligu-valdymas/ uzkreciamosios-ligos/erkiu-pernesamos-ligos/lietuvos-vietoviu-kuriose-uzsikreciama-erkiu-platinamomis-ligomis-zemelapis/* (accessed on 27 February 2024)). Moreover, TBEV was previously detected in questing ticks in the major part of rodent trapping sites [320].

Overall, 139 wild rodents were trapped from March 2019 until May 2020. Wild rodents were captured in live traps using bread soaked in sunflower oil as a bite. The traps were set near the edges of deciduous forests or in open moist areas (in spots where TBEV-infected ticks were previously detected). The summer months were excluded when the trapping of rodents was not productive. Trapped animals were presented to the laboratory on the same day. The species and sex of the rodents were determined according to the species-specific features of the rodents [321, 322]. Six species of rodents were identified: *Apodemus flavicollis*, *Apodemus sylvaticus*, *Apodemus agrarius*, *Mus musculus* (family *Muridae*), *Clethrionomys glareolus* and *Microtus arvalis* (family *Cricetidae*). The decapitation and necropsy of rodents were performed according to standard protocols. Brain and internal organs, such as the spleen, liver, heart, and kidneys were collected from each rodent. In addition, 5 fetal samples were collected. The extracted samples were stored at –80 °C until further analysis.

The data on average monthly air temperature was acquired from the archives of annual reports provided by the Lithuanian Hydrometeorological Service under the Ministry of Environment (*https://www.meteo.lt/category/ menesio-hidrometeorologiniu-salygu-apzvalga/page/5/* (accessed on 27 February 2024)).

2.2. Detection of viral RNA

Pieces (size of 10–100 mg) of the brain (n = 137) and internal organs of each rodent (n = 139) were homogenized individually using a mortar and pestle. Homogenized tissues were mixed with 1000 µL of Modified Eagle's Medium (MEM, Gibco, Waltham, MA, USA) and stored at –80 °C for further

analysis. The brain samples were analyzed separately from the internal organ mix samples collected from the same rodent.

Blood (n = 473) and CSF (n = 3) samples were used after thawing. Each tick was individually treated with liquid nitrogen and homogenized using mortar and pestle. Homogenized tick suspensions were mixed with 1000 μL of MEM and stored at −80 °C for further analysis.

Total RNA was extracted from 300 μL homogenate of wild rodent tissue suspensions and isolates in cell culture, tick suspensions and isolates in cell culture, and blood and CSF samples of dogs. GeneJET RNA Purification Kit (Thermo Scientific, Waltham, MA, USA) was used for RNA extraction according to the manufacturer's instructions.

Extracted RNA from rodent samples was analyzed by real-time reverse transcription polymerase chain reaction (RT-PCR) to detect the TBEV RNA. A reaction mix containing SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Thermo Scientific, Waltham, MA, USA) and primers as described previously were used [323]. TBEV-specific RNA was amplified in 25 μL of a total reaction mixture containing 5 μL sample RNA, 12.5 μL of 2x Reaction Mix, 0.5 μL of SuperScript III Platinum One-Step Taq Mix (Thermo Scientific, USA), 1 μL (300 nM) of forward and 1 μL (900 nM) of reverse primer and 1.5 μL (250 nM) TBE-WT probe. The cycling steps and conditions are described in Table 2.2.1. and primers and probe are presented in Table 2.2.2. All reactions were performed in triplicates.

The whole blood samples of dogs, tick suspensions, and isolates in cell cultures were analyzed by reverse transcription nested polymerase chain reaction assay (RT-nPCR) using DreamTaq Green PCR Master Mix and primers as described previously [324]. The first-step amplification was carried out in 25 μL of a total reaction mixture containing 12.5 μL DreamTaq Green PCR Master Mix (2x), 0.3 μL of RevertAid Reverse Transcriptase (200 U/μL), 0.13 μL of RiboLock RNase Inhibitor (40 U/μL), 0.5 μL of each forward and reverse primers (310 nM), 6.07 μL of nuclease-free water and 5 μL of first-step product (cDNA). The second step of nested PCR was carried out in a total volume of 50 μL of reaction mixture containing 25 μL of DreamTaq Green PCR Master Mix (2x), 1 μL of each forward and reverse primer (260 nM), 18 μL of nuclease-free water and 5 μL of target RNA. The cycling steps and conditions are described in Table 2.2.1. and primers are presented in Table 2.2.2. The selected RT-PCR-positive samples of rodents were prepared for NCR region sequencing as described above. All reactions were performed in triplicates.

For the visualization of the amplified RNA fragment, each PCR product was loaded into a 1.5% TopVision agarose (Thermo Scientific) gel containing

1x TAE buffer and ethidium bromide (5 mg/mL) for 70 min lasting electrophoresis. The GeneRuler 100 bp and 50 bp DNA ladders were used as the molecular size marker. For the sequencing, the obtained products were purified with a GeneJET PCR Purification Kit (Thermo Scientific, USA) according to the manufacturer's instructions.

Table 2.2.1. *Primers and probes used in real-time RT-PCR and nested RT-PCR*

Primers/probe	Primer sequence (5' to 3')	Reference
F-TBE 1	GGGCGGTTCTTCTCC	[317]
R-TBE 1	ACACATCACCACCTCCTTGTCAGACT	
TBE-WT	FAM-TGAGCCACCATCACCCAGACACA-TAMRA	
TBE NS5F	GAGGCTGAACAACTGCACG	[319]
TBE NS5R	GAACACGTCCATTCCTGATCT	
TBE NS5Fn	ACGGAACGTGACAAGGCTAG	
TBE NS5Rn	GCTTGTTACCATCTTTGGAG	
TBE NCR5F	GCGTTTGCTTCGGA	[318]
TBE NCR5R	CTCTTTCGACACTCGTCGAGG	
TBE NCR5Fn	CGGATAGCATTAGCAGCG	
TBE NCR5Rn	CCTTTCAGGATGGCCTT	

Table 2.2.2. *Cycling conditions of real-time RT-PCR and nested RT-PCR*

Target			Real-time RT-PCR			Nested RT-PCR	
			TBE1	NS5	NS5	NCR5	NCR5
Step			One-step amplification	First amplification (cDNA synthesis)	Second amplification	First amplification (cDNA synthesis)	Second amplification
Reverse transcription		temperature	37 °C	42 °C	–	42 °C	–
		time	30 min	30 min	–	30 min	–
Initial denaturation		temperature	95 °C	96 °C	96 °C	96 °C	96 °C
		time	10 min	5 min	5 min	5 min	5 min
Amplification	Cycle count		42	40	40	40	40
	Denaturation	temperature	95 °C	96 °C	96 °C	92 °C	94 °C
		time	15 sec	30 sec	30 sec	1 min	1 min
	Annealing	temperature	60 °C	40 °C	40 °C	37 °C	55 °C
		time	1 min	30 sec	30 sec	1 min	1 min
	Extension	temperature	–	68 °C	68 °C	72 °C	72 °C
		time	–	30 sec	30 sec	1 min	1 min
Final extension		temperature	–	68 °C	60 °C	72 °C	72 °C
		time	–	10 min	10 min	10 min	10 min
Reference			[317]	[319]		[318]	

2.3. Detection of TBEV-specific antibodies

2.3.1. Enzyme-linked immunosorbent assay (ELISA)

The serum samples of dogs were thawed and reached room temperature before analysis. Commercially available quantitative enzyme-linked immunosorbent assay (ELISA) (VetLine TBE/FSME ELISA, NovaTec Immundiagnostica GmbH, Dietzenbach, Germany) kit was used according to the manufacturer's instructions. The kit used is based on the indirect sandwich test principle. Microtiter plates are coated with specific antigens to bind corresponding antibodies in the sample. Conjugated mouse antibodies are used and specifically binded antibodies from the sample are detected by a peroxidase-catalyzed color reaction. The intensity of color was measured by a microplate photometer (Multiskan FC, Thermo Fisher Scientific, Waltham, MA, USA). The intensity of the sample color is proportionate to the quantity of bound antibodies. Samples were considered positive when optical density was above the upper limit of the cutoff value determined by cutoff controls. The levels of antibodies were calculated using a concentration curve obtained from measuring control samples of known concentration.

2.3.2. Virus neutralization test (VNT)

To confirm positive and borderline ELISA results, samples were subsequently tested by the virus neutralization test (VNT) according to the instructions described before [323]. Gold standard in-house neutralization assay was used to neutralize and confirm the presence of TBEV-specific antibodies. Serum samples of dogs were heat-inactivated in a water bath at 56 °C for 30 minutes to degrade the complement proteins. Heat-inactivated samples were diluted in Modified Eagle's Medium (MEM) (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin from 1:5 to 1:320. The diluted serum samples were then incubated with 100 $TCID_{50}$ of the TBEV Neudörfl strain (which was cultivated in Vero (ATCC® CCL-81™) cell culture for 6 serial passages) in a 96-well plate (TPP, Switzerland) for 90 min at 37 °C. The cells were evaluated for the presence of cytopathic effect (shrinkage and detachment of the infected cells) at 3, 5, and 7 d.p.i. using an inverted microscope (Olympus). The highest serum dilution that inhibited the cytopathic effect of the TBE virus was regarded as the end-point titer. Serum samples with a titer of 1:20 and higher were considered to be positive for the presence of anti-TBEV neutralizing antibodies.

2.4. Virus isolation in cell cultures

Suspension samples of rodent tissues were thawed and a total volume of 500 µL of each sample was centrifuged at 12.000× g for 10 min and filtered with a 0.22 µm pore size microfilter (Techno Plastic Products AG, Trasadingen, Switzerland). Murine neuroblastoma cells (Neuro-2a ATCC No. CCL-131) were seeded in 96-well plates and incubated in a cell culture medium containing 1/2 Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) and 1/2 (MEM) supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in 5% CO_2 overnight. Half of the DMEM was replaced with MEM for better adhesion to the surface of the well plates since Neuro-2a cells tend to wash off during the inoculation process. The following day, cells were inoculated with 100 µL of microfiltered supernatant of rodent brain and internal organ mix samples for 90 minutes at 37 °C. Neuro-2a cells were then washed with 1x phosphate-buffered saline (1x PBS) (Gibco, USA) and incubated at 37 °C in 5% CO_2 in 200 µL of DMEM supplemented with 10% heat-inactivated FBS and 100 U/mL penicillin and 100 µg/mL streptomycin.

The suspensions of ticks and blood serum and CSF samples of dogs were centrifuged at 12.000× g for 5–10 min and filtered with a 0.22 µm pore size microfilter. Cell cultures of Vero (ATCC No. CCL-81) and MARC-145 (ATCC No. CRL-12231) were seeded in MEM supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin and incubated at 37 °C in 5% CO_2 overnight. For Neuro-2a cells, 1/2 of MEM was replaced with DMEM. The following day, cells were inoculated with 100 µL of prepared supernatants of the tick suspensions and blood and CSF samples of the dogs for 90 minutes at 37 °C in 5% CO_2. Cells were then washed with 1x PBS and incubated at 37 °C in 5% CO_2 in 200 µL of MEM supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. For Neuro-2a cells, DMEM was used instead of MEM.

Vero and MARC-145 cells were incubated for 7 days and Neuro-2a cells were incubated for 4 days. The cells were monitored for cytopathic effects using an inverted microscope (Olympus). The cells were inoculated in a triplicate setting for each sample including triplicates of positive and negative controls for each round of analysis. Following incubation, the cells were frozen at −80 °C and thawed two times. After 3 serial passages, the cells were frozen at −80 °C and thawed two times, then the cell suspensions were harvested for RNA extraction. Successful virus isolation in cell cultures was confirmed by detecting fragments of TBEV RNA using real-time RT-PCR and nested RT-PCR.

2.5. Viral load quantification

Quantification of the TBEV RNA yield in suspensions and respective cell culture isolates of selected rodents was performed according to the modified method described in a previous study [323]. A synthetic fragment corresponding to the amplified TBEV NS5 region was cloned into the pJET1.2 vector using the CloneJET PCR Cloning Kit (Thermo Scientific, Waltham, MA, USA). A cDNA product of partial NS5 sequence was obtained as described in a previous study [325] and the product band was cut from agarose gel. Used primers are available in Table 2.2.2. and cycling steps and conditions are described in Table 2.2.1. The viral cDNA was extracted from agarose gel using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

The blunting reaction mix was prepared on ice using 10 μL of the 2x reaction buffer, 1 μL of cDNA product, 1 μL of DNA blunting enzyme, and nuclease-free water to reach a total volume of 18 μL. The mixture was incubated for 5 minutes at 70 °C and then cooled on ice. The ligation mixture was prepared by mixing 1 μL of pJET1.2/blunt cloning vector and 1 μL of T4 DNA ligase. Following the cooling of the blunting reaction mix, 2 μL of ligation mixture was added, vortexed for 2−3 seconds, and incubated at room temperature (22 °C) for 5 minutes.

The cloned fragment was then transformed into pretreated *Escherichia coli* cells using the Transform-Aid Bacterial Transformation Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The *E. coli* was cultivated on Mueller-Hinton agar the day before the transformation. C-medium solution was incubated at 37 °C for 20 minutes. *E. coli* in the Petri plates were incubated for at least 20 minutes. T-solution was prepared by mixing T-solution (A) and (B) and the mixture was kept on ice. A single colony (4 × 4 mm) of *E. coli* was placed in 1.5 mL of preheated C-medium solution and incubated at 37 °C for 180 minutes. Following incubation, the C-medium with *E. coli* was centrifuged for 1 minute and the supernatant was removed. Cold T-solution was added to pretreated *E. coli* and incubated on ice for 5 minutes. After incubation, 50 μL of prepared *E. coli* suspension was mixed and kept on ice for 2 minutes and then mixed with the previously prepared cloning mixture kept on ice for 2 minutes. The obtained mixture of transformed cell solution was immediately used and transferred on Mueller-Hinton and MacConkey agar and incubated overnight at 37 °C. All of the cloning and transformation procedures were performed on an ice bath unless it was specified otherwise.

Plasmid DNA was extracted and purified using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, Waltham, MA, USA). The concentration of obtained DNA was quantified using the Qubit dsDNA BR Assay Kit (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. Standard curves for viral load calculations were generated after 10-fold dilutions of stock DNA, which served as templates for quantitative PCR reactions, ranging from 10^2 to 10^8. Quantification was based on real-time RT-PCR using SYBR Green I Dye (Thermo Scientific, Waltham, MA, USA), nested NS5 primers, and cycling conditions as described previously [325] and present in Tables 2.2.1 and 2.2.2 respectively. Sample concentration was calculated using a calibrated standard curve, and viral load was estimated using the following equation:

$$n = \frac{x \cdot 6.0221 \cdot 10^{23}}{(N \cdot 660) \cdot 1 \cdot 10^{9}},$$

where n – is viral copy equivalent count, x – is the amount of amplicon, and N – is the length of dsDNA amplicon.

Ct value of each 10-fold dilution acquired by real-time RT-PCR was assigned a specific viral copy equivalent value distributed along the standard curve. All samples were tested in triplicates, and mean values were calculated.

2.6. Sequencing and phylogenetic analysis

Samples of rodent brain and internal organ mix suspensions positive for TBEV NCR5 were selected for partial gene sequencing. The primer sets that were used for sequencing are presented in Table 2.2.1. A cDNA product of partial NCR5 sequence was established by nested RT-PCR as described in Table 2.2.2. The obtained cDNA product band was cut from agarose gel and then extracted using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The yields of viral cDNA were quantified using the Qubit dsDNA BR Assay Kit (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. Partial gene sequencing targeting the 126 bp NCR region of TBEV was performed using BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Waltham, MA, USA) and 3130 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). Partial NCR sequences of wild rodent-derived TBEV isolated in Lithuania were acquired, although not submitted to the Genbank because the sequencing was used for internal confirmation of the specificity of detected TBEV RNA. The obtained NCR fragments were too short for systematic phylogenetic analysis.

Phylogenetic analysis was carried out by comparing partial NCR fragment sequences from isolates of rodents' brain and internal organ mix samples and previously locally isolated NCR fragments from goat milk and *I. ricinus* ticks, to the reference strains of different TBEV subtypes, including Neudörfl (U27495), Sofjin (AB062064) and Vasilchenko (AF069066). The Omsk hemorrhagic fever virus was used as an outgroup. Sequences of different TBEV strains and closely related flaviviruses chosen from the National Center for Biotechnology Information (NCBI) GenBank database were used for phylogenetic comparisons. The alignments of all sequences used for phylogenetic tree construction were created manually using ClustalW software (Clustal, Dublin, Ireland) in the MEGA X package. The neighbor-joining method was used for phylogenetic tree construction with 1000 bootstrapping replicates.

2.7. Statistical analysis

Binary logistic regression analysis, Chi-square test, and Fisher's exact test were utilized to test the significance of the risk factors. Standardized residuals were employed as post-hoc analysis after statistically significant chi-square tests. The confidence intervals were calculated using the exact binomial method. Linear associations were assessed using the Pearson correlation test. The statistical analysis and visualizations were performed utilizing the programming language R 4.3.2.

2.8. Funding

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2.9. Ethical statement

The trapping of rodents was conducted under the permission of the Environmental Protection Agency and the Ministry of Environment of the Republic of Lithuania (license No. 5 (28 February 2019) and No. 12 (27 February 2020)). All procedures were conducted in compliance with relevant animal welfare guidelines and regulations.

3. RESULTS AND DISCUSSION

3.1. TBEV in dogs

3.1.1. The prevalence of TBEV RNA and TBEV-specific antibodies in blood samples of dogs

In the present study, blood samples of 473 dogs were analyzed by RT-nPCR and ELISA. TBEV RNA was found in 18.6% (88/473; 95% CI 15.2–22.4) of dog blood/serum samples and 2 out of 3 CSF samples (data of anamnesis and outcome of TBE of PCR-positive dogs can be found in Supplementary Material Table S3). The presence of antibodies in 21.6% (102/473; 95% CI 17.9–25.6) of blood serum samples of dogs was revealed. The specificity of detected antibodies for TBEV was confirmed by VNT (Fig. 3.1.1.1 A). The blood samples were collected from dogs presented to the veterinary clinics between May 2020 and December 2021. Although there was a slight tendency to the higher prevalence rate of TBEV-specific antibodies in samples collected in autumn months, no statistically significant associations were found between the prevalence of TBEV and TBEV-specific antibodies in different months (Fig. 3.1.1.1 B). However, since the collection of dog blood samples was organized randomly, the number of analyzed samples was uneven every month, as the number of dogs with possibly TBE-related symptoms, and the presence of recent tick-bite or concurrent other tick-borne diseases.

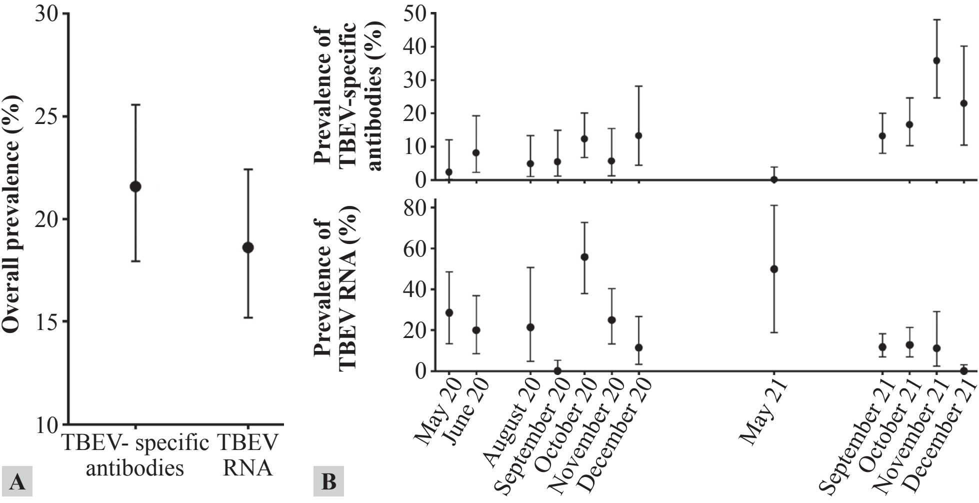

Fig. 3.1.1.1. *The overall prevalence rate (%) of TBEV RNA and TBEV-specific antibodies in blood/blood serum samples of dogs **(A)** and prevalence rate (%) of TBEV RNA and TBEV-specific antibodies in different months of sample collection **(B)***

The supposition of TBE diagnosis is mainly based on TBE-related clinical symptoms, history of recent tick-bite, and visiting/residing in endemic locations. The diagnosis of TBE in human patients is currently confirmed by the detection of TBEV-specific antibodies in CSF or by seroconversion in paired blood serum samples. However, studies in human TBE patients show that TBEV-specific antibodies may still not be present and therefore not detected by serological tests in the early stages of TBEV infection [36]. Moreover, cross-reactions with IgG antibodies of other flaviviruses are prevalent, particularly in locations endemic to several flaviviruses. In human TBE diagnosis, serological testing is notoriously hindered by the cross-reactivity of other flaviviral infections and vaccinations [37]. However, the only possible cross-reactivity in dogs that could interfere with the serological confirmation of TBE is infection with West Nile fever virus [29, 326]. The present investigation showed that only 26.1% (23/88; 95% CI 17.3–36.6) of PCR-positive dogs were TBEV-seropositive. Saksida et al. [327] suggested that PCR is an efficient method for early diagnosis of TBEV infection when TBEV-specific antibodies in the blood serum or CSF are not present and detectable yet. However, TBEV RNA detection by PCR is rarely utilized to confirm TBE infection in humans or dogs because of the rapid virus clearance from the organism in the main part of clinical cases when TBE-specific symptoms develop. Although Schultze et al. [328] concluded that to make diagnostics more specific and to improve patient management, the detection of viremia in the first phase of the biphasic course of TBE (which can be suspected after a tick bite followed by feverish illness accompanied by thrombocytopenia and leukocytopenia) would enable early diagnosis and prediction of the second phase of TBE, developing in major part of patients.

The TBEV RNA prevalence of 18.6% and seroprevalence of 21.6% in randomly collected dog blood serum samples revealed in the present study correspond to the results reported in other European studies. A similar study in a TBEV endemic region in Czechia reported a TBEV RNA prevalence rate of 12.6% (20/159) and seroprevalence of 11.3% (18/159) in randomly collected blood samples of dogs [329]. The results of the present study and the study mentioned above suggest that TBEV RNA detection by PCR might be beneficial in diagnosing infection in the initial or acute phases, particularly in endemic countries.

The present study reported neurological symptoms in 32.1% of TBEV-seropositive dogs. Only a few studies analyzed the prevalence of TBEV-specific antibodies in dogs with neurological symptoms. A high seroprevalence was reported in Germany, reaching 53.6% in blood serum and 43.6% in CSF samples [330]. Similar to the present study, a TBEV-seroprevalence of 20.2% in dogs with neurological symptoms was detected in Austria [316].

A high TBEV-seroprevalence was reported in healthy dogs in Europe. Several studies reported similarly high seroprevalence of TBEV-specific antibodies, reaching 40% in the Åland Islands of Finland [331], 30.4% in endemic regions of Germany [330], and 30.4% in Denmark [332]. However, the ELISA results were confirmed by VNT only in the study conducted on the Danish Island. The confirmed seroprevalence was substantially reduced to 4.8%, and TBEV-specific antibodies were detected only in the Danish Island Bornholm in the Baltic Sea [332]. The discrepancy in ELISA and VNT results implies that the actual seroprevalence could also be reduced if VNT confirmation were applied in the other two studies mentioned above. A seroprevalence of TBEV-specific antibodies in 6.7% was reported in healthy dogs in Finland [331], 3.3% in Czechia [314], 2.1% in Germany [333], 1.7% in Spain [334], and 0.1% in Belgium [335]. The high seroprevalence of TBEV-specific antibodies detected in the present study indicates that TBE in dogs in Lithuania is a common tick-borne pathogen, similar to other TBEV-endemic areas.

The present study revealed the rate of death/euthanasia in 18.2% (16/88; 95% CI 10.8–27.8) of PCR-positive dogs. The presence of severe neurological symptoms or poor prognosis of disease outcome were the reasons for death/euthanasia in 12.5% (11/88; 95% CI 6.4–21.3) of PCR-positive dogs. The remaining 5.7% (5/88; 95% CI 1.9–12.8) of PCR-positive dogs died regardless of applied symptomatic treatment and intensive care. A death/euthanasia rate of 18.2% observed in the present study is substantially lower than the rate of 33.3% found by Kleeb et al. [30] in Switzerland. However, the present study analyzed randomly collected dog blood serum samples. Meanwhile, Kleeb and colleagues [30] retrospectively analyzed the data of serologically confirmed TBE cases in dogs with clinical neurological symptoms. The rate of mortality in humans is estimated at 1−4% in Europe and 15−20% in areas where TBEV-FE is prevalent [275, 336]. In addition, between 30−50 % of clinical TBE cases may develop into severe neurological disease and long-term sequelae in human patients [337].

The present study revealed the seroprevalence of TBEV-specific antibodies in 26.1% (23/88; 95% CI 17.3−36.6) of PCR-positive dogs (mean level 189 NTU/mL; range 29.7−525 NTU/mL). The median age of PCR-positive dogs carrying TBEV-specific antibodies was 8 years (1 to 14 years). Dogs older than 1 year of age were found to be TBEV-seropositive and at the same time PCR-positive for TBEV RNA significantly more often ($p < 0.01$) compared to dogs under the age of 1 year (37.5% (95% CI 26.3−49.7) and 0% respectively). However, a low (n = 7) number of PCR-positive dogs under 1 year of age needs to be considered. The most presumable argument to explain this finding is the first-time TBEV infection in these juvenile dogs.

Therefore, TBEV-specific antibodies were not present or detectable because of the initial phase of the TBE infection at the time of testing. A statistically significant correlation between the age of dogs and the seroprevalence of TBEV was revealed in a study in Czechia [313]. However, the reported data on TBEV seroprevalence in dogs under 1 year of age is limited. Older dogs are primarily included in the TBEV seroprevalence studies, because of the higher probability of former contact with ticks and as a result, with tick-borne pathogens, including TBEV [313, 332]. In addition, dogs of small breeds (less than 15 kg weight) were excluded from some TBEV-specific antibody seroprevalence investigations for the same purpose as juveniles [332]. Nonetheless, the results of the present study demonstrate that the infantile age or small-scale size of the dog should not be the reason to reject TBE as a differential diagnosis, particularly in TBEV endemic areas. However, suspected TBE cases in six dogs were reported in the United Kingdom [338], where TBEV presence in ticks (and potentially humans) has been detected only recently [82, 339].

In 29.6% (26/88) of PCR-positive dogs recent tick bites (in the past two weeks) were reported by owners. Coinfection with other tick-borne pathogens was diagnosed in 19.3% (17/88; 95% CI 11.7–29.1) of PCR-positive dogs. Protozoa of *Babesia* spp. were found in the erythrocytes of 82.4% (14/17) of PCR-positive dogs. Morulae of *Anaplasma* spp. were found in the neutrophils of three (17.6% (3/17)) PCR-positive dogs. In addition, *Borrelia burgdorferi*-specific antibodies were simultaneously detected in one dog with anaplasmosis and TBE. As far as we know, this is the first report of TBE and active *Anaplasma phagocytophilum* coinfections simultaneously diagnosed and reported in dogs. However, clinical TBE cases in dogs with high antibody levels against *A. phagocytophilum* and *Ehrlichia canis* in blood serum were reported previously in Austria [317] and Greece [340]. Concurrent TBE and *Babesia* spp. infections were previously reported by Bajer et al. in a sled dog from Poland after a visit to Estonia [34]. The results of the present study highly suggest that coinfection of tick-borne pathogens in dogs might be underdiagnosed, primarily in locations where several tick-borne pathogens cocirculate.

The present study detected TBEV RNA in dogs of 36 different breeds. The most commonly infected were mixed breed dogs (23.9% (21/88)), followed by German shepherds (11.4% (10/88)), Yorkshire terriers (9.1% (8/88)) and Akita’s 9.1% (6/88)).

The present study found TBEV-specific antibodies in 16.7% (79/473) of dogs without TBEV RNA in blood samples.

3.1.2. Risk factors of severe course of TBE and lethal outcome in dogs

Out of 88 PCR-positive dogs, TBEV RNA was found in the blood samples of 48 male and 40 female dogs. The median age of PCR-positive dogs was 7 years (from 2 months to 15 years). The present study significantly more often ($p < 0.01$) observed neurological symptoms in male dogs (43.7% (95% CI 29.5−58.8) compared to females (17.5% (95% CI 7.3−33.0). Moreover, the older age of the dog was associated with a higher risk of the lethal outcome/euthanasia of PCR-positive dogs showing neurological symptoms. The mean age of recovered dogs was 5.6 years and the mean age of dead/euthanized dogs was 11.8 years ($p < 0.01$). Other causes for death/euthanasia were not associated with the age of PCR-positive dogs. Furthermore, dogs who showed neurological symptoms were more likely to face a worse outcome of the TBE disease ($\chi^2 = 11.57$, df = 2, $p < 0.01$). The number of recovered dogs who showed neurological symptoms was significantly lower (27.8% (95% CI 19.3−36.6)) compared to the number of recovered dogs who did not show any neurological symptoms (72.2% (95% CI 63.7−80.7)). A retrospective study by Kleeb and colleagues [30] analyzed the risk factors of dogs with confirmed TBE and reported that older dogs and dogs who experienced seizures had an increased risk of a lethal outcome, similar to what was observed in the present study. An older age and male gender are associated with a more severe course of TBE infection in human patients [297, 298].

A statistically significant ($p < 0.05$) association between the seroprevalence of TBEV-specific antibodies in the blood serum of PCR-positive dogs and the outcome of the TBE ($\chi^2 = 5.05$, df = 1). Subsequent post-hoc analysis showed that compared to the predicted model findings, the rates of recovery from TBE of PCR-positive dogs were lower by 14.2% in the TBEV-seropositive group and higher by 8.6% in the TBEV-seronegative group. On the other hand, rates of death/euthanasia were 65.5% higher in the TBEV-seropositive group and 39.7% lower in the TBEV-seronegative group of PCR-positive dogs. Higher rates of death/euthanasia in viremic seropositive dogs indicate that the delayed or insufficient production of TBEV-specific antibodies might be related to a higher risk of lethal outcomes in dogs with acute TBE. Based on data in human patients, viremia is generally short-lived and TBEV is no longer detectable at the onset of neurological symptoms and humoral response [327]. Studies in human patients conclude that the delayed immune response and production of TBEV-Ig antibodies is the predictor factor of the severe course of TBE infection [341, 342]. The monophasic course of the TBE [277, 308] and comorbidities [308] were also associated

with a more severe course of TBE. In addition, some studies suggest that previous immunization not protecting an individual may negatively affect the outcome [342, 343]. The phenomenon named antibody-mediated enhancement of viral infection is well-described and has been reported in many viral infections, including flaviviral and TBE [344]. *In vitro* studies suggest that the enhancement may be TBEV complex-specific, rather than flavivirus-specific [345].

3.1.3. TBEV seroprevalence and relations with clinical symptoms and the prevalence of concurrent tick-borne diseases

Overall, 13.7% (14/102) of blood serum samples of dogs showed borderline results (20−30 NTU/mL) by ELISA test, and 86.3% (88/102) were found to be positive (> 30 NTU/mL) (Fig. 3.1.3.1 A–C). The VNT confirmed the specificity of detected antibodies to TBEV. Significantly higher TBEV-specific antibody levels were detected in blood serum samples of dogs with TBE-related symptoms in comparison to dogs who showed non-specific ($p < 0.01$) and dogs who had non-TBE-related symptoms ($p < 0.01$) (Fig. 3.1.3.1 D). Most likely these dogs could have had a recent asymptomatic TBEV infection. On the other hand, misdiagnosis with other neurological diseases is also possible. Few reports of TBE in dogs suggested that TBE might be underdiagnosed in dogs considering mild or nonspecific clinical symptoms or unawareness of the probable infection in severe clinical cases when TBE-related neurological symptoms are present [29, 32]. As summarized by Weissenböck et al. [318] TBE must be included in the list of nonsuppurative encephalitis in dogs, especially in TBEV-endemic locations. The TBE should be distinguished and differentiated from encephalitis of other defined etiologies (e.g., rabies, Aujeszky's disease, canine distemper) and many still etiologically undefined cases of nonsuppurative encephalitis of dogs [318].

Concurrent tick-borne infections were diagnosed in 14.7% (15/102) of dogs with TBEV-specific antibodies (13 dogs had *Babesia* spp. and 2 had antibodies specific to *Anaplasma* spp.). Moreover, significantly ($p < 0.05$) higher levels of TBEV-specific antibodies were found in dogs diagnosed with concurrent tick-borne infections (Fig. 3.1.3.1 E). These results imply that dogs with a history of previous tick-borne infections are most likely residing in or visiting localities where ticks are prevalent and therefore have a higher risk of tick-bite and tick-borne infections. Furthermore, a 50−100 times higher risk of TBEV-infected tick bite in dogs set side by side with humans has been estimated [29]. In addition, the ownership of the dog and walks more often than four times in a week were reported as risk factors for human TBE (odds ratio (OR) = 2.45 and 2.11, respectively) in a study conducted in Germany [346].

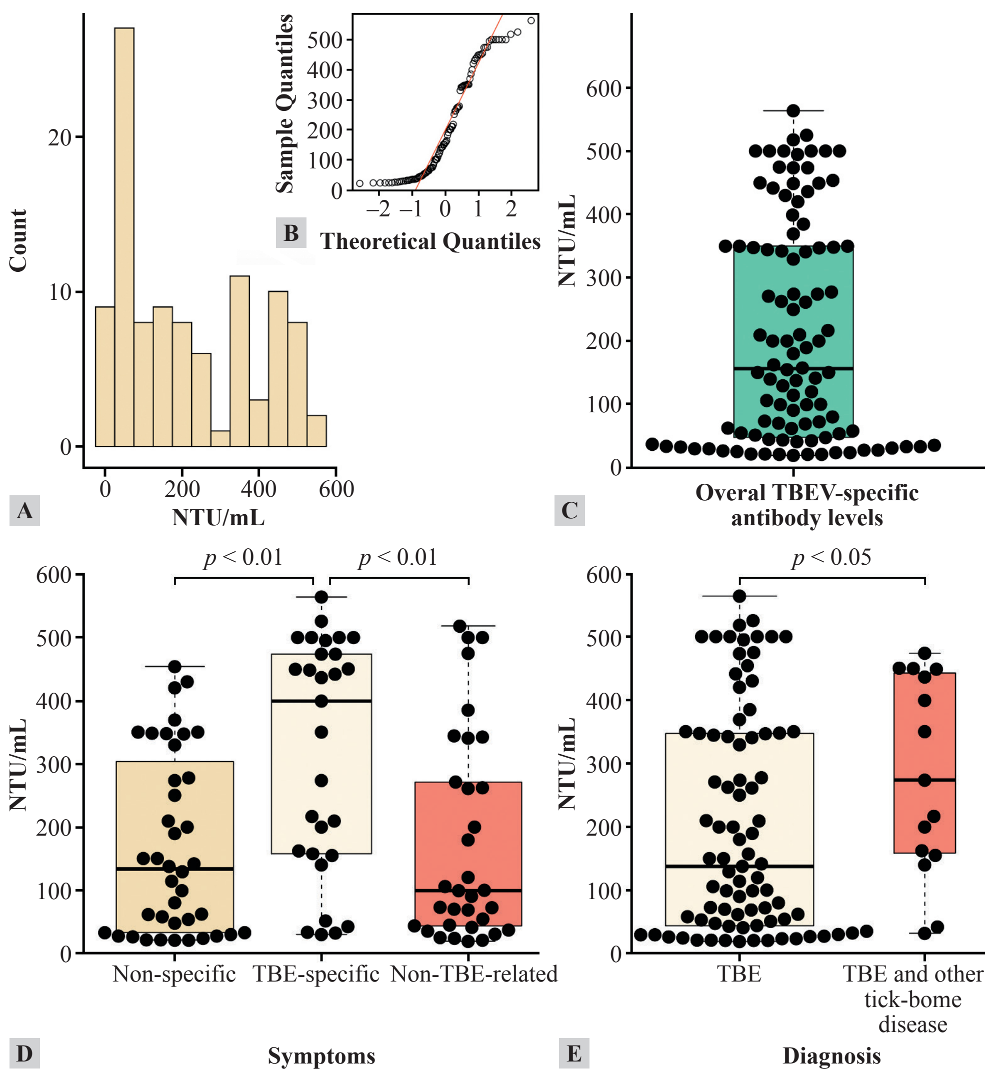

***Fig. 3.1.3.1.** Number of seropositive dogs and levels of detected TBEV-specific antibodies **(A)**. Theoretical quantiles **(B)**. Differences in levels of TBEV-specific antibodies in seropositive dogs **(C)**. Differences in levels of TBEV-specific antibodies in dogs with potentially TBE-specific symptoms, non-specific symptoms, and dogs with non-TBE-related symptoms **(D)**. Differences in levels of TBEV-specific antibodies in dogs with concurrent tick-borne infections and dogs without concurrently diagnosed other tick-borne diseases **(E).** Figures C–E: Black dots represent seropositive dogs. Confidence intervals are visualized by the figures of different colors and the black horizontal lines in the figures show the average antibody levels in each group of dogs*

Clinical TBE-related neurological symptoms were found in 13.7% (14/102) of TBEV-seropositive dogs. In 32.4% (33/102) of dogs with TBEV-specific antibodies in blood serum, non-specific symptoms like fever, apathy, and symptoms of gastrointestinal system complaints (vomiting and diarrhea) were observed. Other infections and illnesses were diagnosed in 39.2% (40/102) of TBEV-seropositive dogs with various symptoms considered not related to TBE (diseases and infections of skin, uterus, kidney or liver failure, trauma, oncologic processes, etc.).

3.1.4. Virus isolation from PCR-positive dogs' serum and CSF samples

Isolation of the TBE virus in cell cultures from blood or CSF samples is rarely utilized principally because of a short-lived TBE viremia [313]. TBEV was successfully isolated from blood serum (n = 7) and CSF (n = 2) samples of PCR-positive dogs in the present study. Neuro-2a, Vero, and MARC-145 cell lines were utilized to isolate the virus. A successful isolation of TBEV after the third serial passage in cell culture was confirmed by PCR.

3.1.5. Clinical symptoms of TBEV RNA-positive dogs

Neurological symptoms were observed in 31.8% (28/88) of PCR-positive dogs. Moreover, TBEV-specific antibodies were detected in the blood serum of 32.1% (9/28) of PCR-positive dogs with neurological symptoms. The absence of TBEV-specific antibodies in the blood serum of the remaining dogs may suggest two possible reasons: early phase of TBE infection or acute course of the disease. In line with the results of the present study, the analysis of dogs in the TBEV-endemic region of Czechia observed neurological symptoms in 35% (7/20) of PCR-positive dogs [329]. Bogovič et al. [347] analyzed the clinical symptoms of viremic patients who had TBEV RNA in their blood but did not show symptoms of CNS involvement. The study mentioned above reported that 98% of patients showed malaise or fatigue, 85% had headaches, and 54% had myalgias [347]. Mild neurological symptoms observed in PCR-positive but TBEV-seronegative dogs in the present study could be associated with the symptoms of the initial phase of TBE. Similarly, studies in human patients show that the production of TBEV-specific antibodies may be deliberate or depressed in cases of severe TBE course [348].

The mean age of the dogs with neurological symptoms was 7.84 years, ranging from 4 months to 15 years. Reports in human TBE cases show that the youngest patient with clinical TBE was 17 days old and unfortunately, with severe clinical symptoms and sequelae [349]. However, the major part

of clinical TBE cases in infants and children are milder and the risk of sequelae is lower compared to adult patients [350]. Moreover, Salat et al. [351] suggested that it is likely that the age and breed of the dog, health and immune system status, specific TBEV strain, and infectious dose of the virus play an important role in predisposing the clinical symptoms and severe course of TBE.

Severe or moderate neurological symptoms were observed in 12.5% (11/88) of PCR-positive dogs. Six viremic dogs showed episodes of seizures. Three viremic dogs concurrently showed symptoms of meningoencephalo-myelitis – episodes of vision impairments, deficits of fascial reflexes, and tetraparesis. Two viremic dogs showed isolated seizures and one dog concurrently had altered mental status among the episodes of seizures. Moreover, out of six dogs with seizures five dogs had lethal outcomes and four of those dogs did not show hyperthermia. Although the number of dogs with seizures is insufficient to conclude accurately, this observation implies that the absence of hyperthermia in the presence of seizures might be related to a poor outcome of TBE. In line with the present study, a retrospective analysis of clinical TBE in dogs concluded that hyperthermia was linked to better outcomes of TBE [30]. The owners of four PCR-positive dogs reported changes in the dog behavior. Two dogs were anxious and hyper-excited/ hypersensitive at night, one dog was redundantly vocalizing without an obvious reason and one dog showed cognitive function impairment. Additio-nally, one PCR-positive dog was nonresponsive to external stimuli and showed altered mental status.

In the retrospective TBE study in dogs by Kleeb and colleagues [30], 31% (n = 17) of dogs developed seizures (cluster seizures in six, focal seizures in four, status epilepticus in four, and isolated epileptic seizures in three dogs), which indicates that seizures are a common clinical symptom in dogs with severe or moderate clinical course of TBE. On the other hand, focal or generalized seizures are an infrequent symptom of tick-borne encephalitis in humans, who most often have meningitis or encephalitis [4]. However, meningoencephalitis with seizure activity and fever were reported in a few reports of clinical TBE in infants [349, 352−354]. However, TBE is usually manifested as meningitis and therefore has a milder course of the disease and lower risk of sequelae in children and adolescents compared to TBE in adults [275].

In a retrospective study of clinical TBE in dogs, clinical neurological syndrome at presentation was consistent with meningoencephalitis in 42%, meningomyelitis in 33%, meningoencephalomyelitis in 19%, and meningitis in 5% of dogs [30]. A substantially different picture of neurological symp-toms is seen in human patients. The TBE manifests as meningitis in 50% of

patients, meningoencephalitis in 40%, and meningoencephalomyelitis in 10% of clinical TBE cases in Europe [275].

Mild neurological symptoms were observed in 19.3% (17/88) of PCR-positive dogs. Symptoms of walking impairment, reluctance to walk, stiff walking, difficulty standing up, hyperesthesia in the back area (without organic defects in the musculoskeletal system), and coordination impairment were present in PCR-positive dogs.

No neurological symptoms that could potentially be related to TBE were observed for the rest of the 68.2% (60/88) of PCR-positive dogs. A quarter (25% (22/88) of PCR-positive dogs were diagnosed with various concurrent diseases. Symptoms of gastrointestinal system impairment (vomiting/diarrhea) were observed in 37.5% (33/88) of viremic dogs (of them, 17% (15/88) of dogs simultaneously had neurological symptoms). Concurrent tick-borne diseases were diagnosed in 13.6% (12/88) of dogs (excluding five dogs with neurological symptoms). Non-specific symptoms like apathy and food aversion were present in 11.3% (10/88) of viremic dogs.

Hyperthermia was present in 37.5% (33/88) of viremic dogs: 15.9% (14/88) were diagnosed with concurrent tick-borne disease, 13.6% (12/88) of hyperthermic dogs had TBE-related symptoms, and the rest 10.2% (9/88) of viremic dogs had other likely causes for hyperthermia (trauma, neoplastic processes, etc.).

The present study revealed similar clinical TBE symptoms of PCR-positive dogs to those reported in several European studies [30, 34, 316−318]. However, vision impairment caused by optic neuritis or other ocular reasons is a rare clinical symptom of TBE in dogs or humans, although two cases have been previously described in dogs [34, 317]. In the present study, four dogs had transient vision impairment/loss. Three viremic dogs and one PCR-negative but TBEV-seropositive (with high levels of the TBE-specific antibodies and neurological symptoms) showed transient vision impairment which improved after treatment with corticosteroids. This observation suggests that in endemic areas TBEV should be considered one of the probable causes of immediate and transient vision impairment in dogs, and therefore included in the differential diagnostic list. Although many tick-borne pathogens can be related to vision impairment, no cases of optic neuritis caused by TBEV in humans were reported [355]. However, a case of TBE-related uveitis, that caused transient (resolved after treatment) unilateral vision loss was reported [356]. Moreover, few studies suggested that intermediate uveitis might be triggered by vaccination against TBEV [357−359].

In the present study, 37.5% (95% CI 27.4−48.5) of PCR-positive dogs had symptoms of gastrointestinal system impairment, such as vomiting and diarrhea. Gastrointestinal symptoms were prevalent in 44.1% of dogs with

clinical TBE [30]. In a few studies of TBE in human patients, similar prevalence rates of gastrointestinal symptoms were reported. A survey of human TBE clinical cases with neurological complications conducted by Zambito Marsala et al. [360] reported vomiting in 30% and nausea in 24.7% of patients. A study by Mickienė et al. in Lithuania reported gastrointestinal complaints in 21.3% of TBE human patients [280].

3.1.6. Clinical symptoms of TBEV-seropositive dogs

Neurological symptoms were present in 12.7% (10/79) of TBEV-seropositive, but not viremic dogs. Two seropositive dogs expressed significant changes in behavior. One dog was found by its owners in a hypersensitive state; the dog was hyperactive, disorientated, and had injured himself. The episodes of hypersensitivity lasted for a few days. Moreover, a transient vision impairment was observed. Another dog showed hyperactivity and anxiety and had unusual episodes of extremely increased appetite for grass eating and carpet licking. High TBEV-specific antibody levels (473 NTU/mL and 494 NTU/mL respectively) were detected in the blood serum of both dogs and both dogs completely recovered. Most likely these dogs had a clinical TBE disease because of very high levels of TBEV-specific antibodies in blood serum and TBE-related clinical symptoms which had no other obvious reasons and the presence of changed behavior was sudden. Mild neurological symptoms (difficulty standing up, reluctant, stiff or impaired walking, hyperesthesia in the back area) were present in 8.9% (7/79) of TBEV-seropositive and not viremic dogs and one dog showed uncoordinated movements and episodes of falling.

No potentially TBE-related symptoms were observed in 48.1% (38/79) of TBEV-seropositive nonviremic dogs who were presented because of various health issues or for prophylactic tests. Nonspecific symptoms (apathy and loss of appetite) were present in 29.1% (23/79) of dogs who had no final diagnosis and symptoms of gastrointestinal system function impairment was observed in 26.6% (21/79 dogs) of TBEV-seropositive nonviremic dogs.

3.2. TBEV in ticks, collected from dogs

Attached ticks (n = 117) were detached and collected from the dogs who were analyzed for the prevalence of TBEV and TBEV-specific antibodies in the present study. Suspensions of each tick were prepared separately and analyzed by RT-nPCR and concurrently inoculated on Neuro-2a, Vero, and MARC-145 cells. The TBEV RNA was found in 34.2% (40/117) of tick suspension samples. The number of TBEV-positive tick samples increased

significantly ($p = 0.01$) to 56.4% (66/117) in MARC-145, 58.1% (68/117) in Vero, and 60.6% (71/117) in Neuro-2a cells following three serial passages (Fig. 3.2.1 A, B).

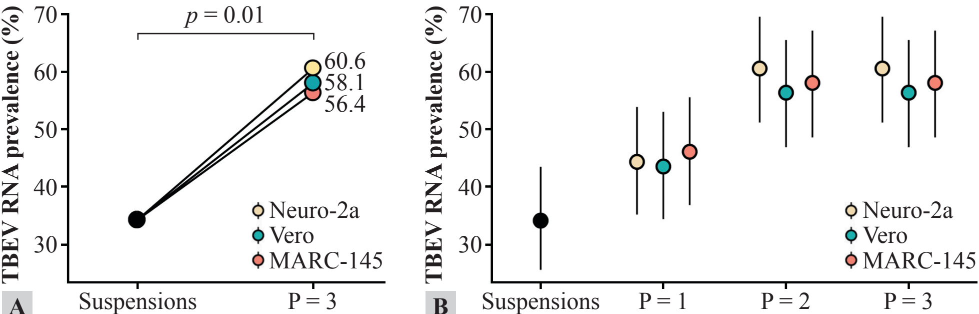

Fig. 3.2.1. *Comparison of the prevalence rate (%) of TBEV RNA in tick suspensions and third passage isolates in Neuro-2a, Vero, and MARC-145 cell cultures* ***(A)****. TBEV RNA prevalence rate (%) changes at each passage in different cell cultures* ***(B)***

The present study shows that TBEV loads in ticks might be too low to be detected by PCR. The prevalence of TBEV RNA in questing ticks is mostly analyzed in pooled tick samples, therefore minimal infection rates (MIRs) are usually reported [85]. In previous European studies, the minimal infection rate in questing ticks was commonly less than 1%, reaching 0.45% in nymphs and 1.05% in adult ticks [8, 156, 361, 362]. However, in a few studies in TBEV endemic regions in Europe, it has reached 2.6−9% in *I. ricinus* [79, 363] and 10.8% in *D. reticulatus* ticks [364].

An experimental study by Belova et al. [365] suggests that TBEV load increases when ticks are fed blood. However, contradicting results were reported in questing and fed ticks in Latvian and Russian studies. A study conducted in neighboring Latvia reported a prevalence of TBEV in questing *I. ricinus* ticks between 1.7% and 26.6% in 2001 and 1995, respectively. The prevalence of TBEV RNA in ticks detached from humans was even higher, between 13.2% and 40.9% in 1997 and 1999, respectively [366]. In contrast, a study in Russia [367] reported a higher TBEV prevalence in questing ticks (10.9–38.7%) compared to ticks detached from humans (5.5–11.0%). However, it is important to note that different methods of TBEV detection were used in the studies mentioned above. The prevalence of TBEV was revealed following the isolation in porcine kidney (PK) cells and confirmed by direct immunofluorescence test (and additional tests) in questing ticks. On the other hand, TBEV was detected by indirect immunofluorescence assay in ticks removed from humans [367]. The results of the present study and the study

mentioned above show that TBEV isolation in cell culture is more competent to increase TBEV viral load in tick samples to detectable levels compared to replication in the cells of ticks' salivary glands during a blood meal.

No statistically significant relations between TBEV presence in dogs and ticks, detached from respective dogs were detected in the present study. A few possible reasons might have influenced these findings. One possible explanation is that the dogs could have been infected with TBEV by ticks not included in the present study. Moreover, the duration of the tick's blood meal or the TBEV load in the attached ticks could be insufficient to infect some of the dogs [368]. A human study reports that the reactivation (required for some microorganisms before the infectivity is attained) might not have enough time to progress since most people remove ticks quickly (in around 90% of cases) [367]. Other factors, such as the response of the host's immune system might influence the success of virus replication. A study conducted in Sweden investigated the clinical symptoms and antibody responses of tick-bitten humans and the prevalence of TBEV in the ticks detached from respective participants. TBEV-specific antibodies were detected in two participants, one of them reported clinical symptoms. Interestingly, TBEV was not detected in detached ticks. Contrarily, no clinical symptoms and seroconversion were detected in one participant, despite the detached tick containing TBEV (1800 copies) [368].

3.3. TBEV in wild rodents

3.3.1. The prevalence of TBEV RNA in wild rodents

The present study analyzed 139 small wild rodents, captured in 19 different TBEV foci in Lithuania. Suspension samples of the brain (n = 137) and a mix of internal organs (heart, spleen, kidney, and liver) (n = 139) were analyzed by real-time and nested RT-PCR. The results revealed a very high TBEV RNA prevalence in wild rodents, captured in known TBEV foci. TBEV RNA was detected in at least one sample of 104 (74.8%; 95% CI 66.7–81.1) wild rodents. Viral RNA was detected in 51.1% (95% CI 42.4–59.7) brain suspensions and 53.2% (95% CI 44.6–61.7) internal organ mix suspensions of wild rodents. The specificity of obtained RNA fragments in PCR-positive samples was confirmed by partial genome sequencing targeting the 126 bp NCR region (Supplementary Material Fig. S1).

After sample cultivation in murine neuroblastoma Neuro-2a cell culture, the viral RNA detection rate increased significantly ($p < 0.01$) (Fig. 3.3.1.1). A total number of 134 (96.4%; 95% CI 91.8–98) positive rodents was revealed after virus isolation in neuroblastoma cells. TBEV RNA was found

in Neuro-2a cell culture of 85.4% (95% CI 78.4–90.9) of brain sample isolates and 81.3% (95% CI 73.8–87.4) of internal organ mix sample isolates. Moreover, TBEV RNA was detected in rodent samples (n = 30), considered negative before sample cultivation in Neuro-2a cells.

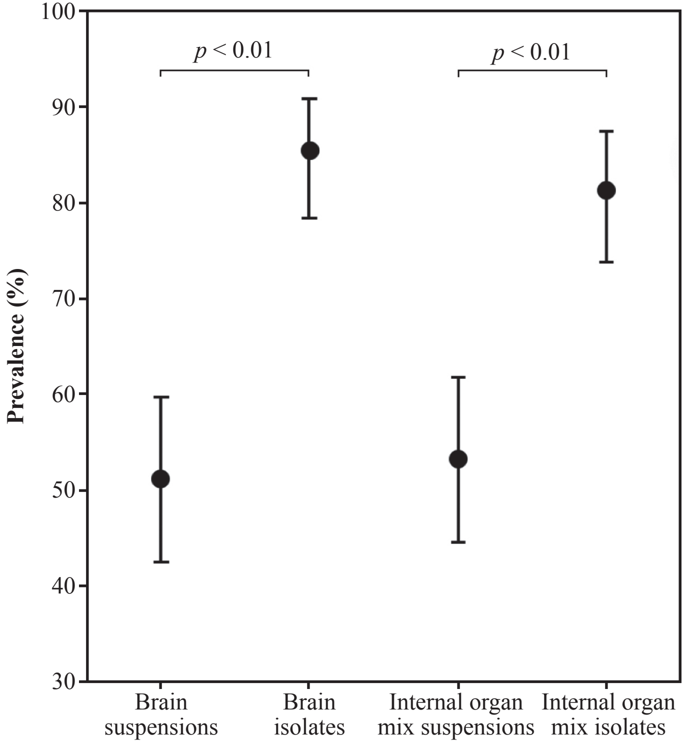

Fig. 3.3.1.1. *The prevalence rate of TBEV RNA in wild rodent brain and internal organ mix samples before (suspensions) and after (isolates) cultivation in murine neuroblastoma (Neuro-2a) cell culture*

The prevalence of TBEV RNA in rodents after sample cultivation in murine neuroblastoma cells suggests that the main part of wild rodents captured in confirmed TBEV foci in Lithuania were carrying the TBEV. In line with the results of the present study, Bakhvalova et al. [25, 369] have previously conducted two separate studies in Siberia's TBEV-Sib endemic regions and suggested that most wild rodents and insectivores might be carrying the TBEV-Sib. The prevalence of TBEV-Sib RNA was detected in 61.4% (±5.3%) by PCR in one of the studies. Moreover, TBE viral antigen in 60.3% (±6.2%) of rodent brain and spleen samples was found by ELISA [25]. In a more recent study, TBEV-Sib RNA was detected by PCR in 62.1% (±3.2%) of rodent brain and spleen samples and increased to 70.9% (±3.0%) after TBEV

protein E was revealed in an additional 8.8% PCR negative samples using ELISA for TBEV antigen detection [369]. However, in the present study, TBEV-Eu subtype RNA prevalence in small wild rodents was investigated and such studies in Europe are scarce. In high-risk areas in Hungary, TBEV-Eu RNA was found in 4.2% (17/405) of liver samples of rodents collected over 7 years [370]. In Germany, TBEV RNA was detected in 15% (21/137) of brain and spleen samples of rodents, collected in TBEV-risk areas and 8% (24/304) of rodents captured in non-risk locations [371]. A study conducted in Finland investigated the prevalence of TBEV RNA in the brain, spleen, and other internal organs of wild rodents collected in late winter two years in a row in two separate TBEV-Sib and TBEV-Eu endemic areas [372]. A prevalence rate of 16.8% (16/95) in TBEV-Eu endemic and 6.3% (5/80) in TBEV-Sib endemic zone-collected wild rodents was revealed. Moreover, TBEV-Eu RNA was detected in 54.2% (13/24) of brain samples of *Microtus agrestis* voles collected in 2009, and TBEV-specific antibodies were detected only in 8.3% (2/24) of PCR-positive rodents. The abovementioned study's results also support the suggestion of virus persistence in rodents in a latent infection even without detectable TBEV-specific antibodies [372]. In addition, TBEV-Eu was detected in samples of lungs and spleens of 20.8% (5/24) *A. agrarius* rodents captured in Korea [70].

However, most TBEV monitoring studies in small wild rodents captured in TBEV endemic areas in Europe analyzed the prevalence of TBEV-specific antibodies. The overall highest antibody prevalence was reported in 61.4% of rodents in Siberia where TBEV-Sib and TBEV-Fe are prevalent [25]. The highest antibody prevalence in Europe reaching 16.9% in rodents was reported recently in a study conducted in Germany [373]. The prevalence of TBEV-specific antibodies reaching 14.8% was revealed in Poland [374], 14.6%, and 7.0% in two separate studies in Slovakia [141, 375], 14.0%, and 10.2% in Germany [371, 376], 5.9% in Slovenia [22], 5.1% in Hungary [377], 4.2% in France [378], 4.0% in Finland [372] and 3.6% in Switzerland [379]. However, the investigations mentioned above utilized different methods, and not all included VNT to avoid false-positive results caused by cross-reactivity with antibodies of other flaviviruses.

In the present study, TBEV RNA was detected in all 19 TBEV foci collected samples of rodents (Fig. 3.3.1.2). Based on PCR results in suspension samples of rodent brain and internal organ mix TBEV RNA prevalence rates ranged from 25 to 100% (Fig. 3.3.1.2 A). The detection rate of TBEV increased significantly ($p < 0.01$) following sample cultivation in murine neuroblastoma Neuro-2a cells. The prevalence rate of 100% was revealed in 16 TBEV foci, and 71.42–90% in the remaining 3 foci (Fig. 3.3.1.2 B).

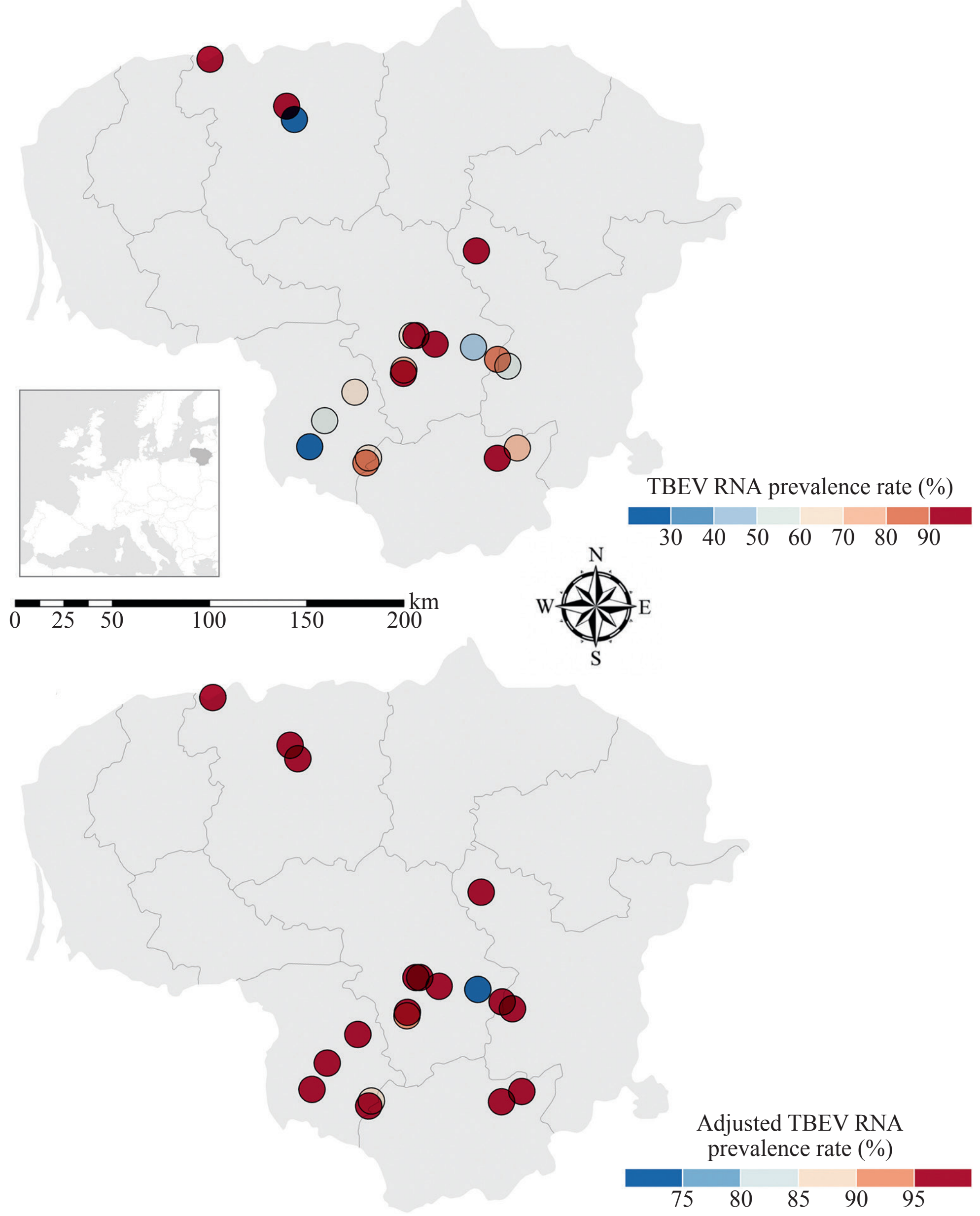

Fig. 3.3.1.2. *Spatial distribution of sample collection sites and average TBEV RNA prevalence rate before* ***(A)*** *and after* ***(B)*** *isolation in murine neuroblastoma Neuro-2a cells in different trapping locations in Lithuania. Viral RNA prevalence rate (%) in wild rodents is indicated by different colors shown in legends*

A limited number of studies have investigated the prevalence of TBEV RNA in wild rodents, therefore the probable key influencing factors are hard to predict. The variety of TBEV RNA prevalence rates in internal organs and brains of rodents collected in TBEV endemic and even nonendemic areas is high. Such differences indicate that many factors might play a role in affecting the detection of TBEV RNA. Several factors might have contributed to the high TBEV RNA prevalence rate in small wild rodents captured in TBE foci in Lithuania which was revealed in the present study.

First of all, the specific sites of rodent trapping were selected carefully based on data from 2016 to 2018 of known TBEV foci related to human TBE cases and probable locations of infected tick-bite, and most importantly – based on the detection of viral RNA in ticks in the majority of sampling sites [320]. Borde and colleagues [380] suggested that the TBEV transmission might circulate in small areas (average size of about 0.5−1 ha) of so-called microfoci, which are stable and usually do not expand or shift for decades. Other authors suggest that the micro-foci might be embedded in a larger defined natural focus [381−383]. Moreover, Danielová et al. [384] reported the expansion of TBEV foci to higher altitudes. Bournez et al. [385] reported a disappearance of TBEV foci in France, indicating that the dynamics of virus circulation in nature are still unclear. However, the present study suggests that capturing rodents in microfoci might increase the chances of viral RNA detection.

Secondly, the TBE infection rate in the human population in Lithuania has been the highest in Europe for more than the past ten years. The infection rate has been considerably higher compared to other European countries where the prevalence of TBEV RNA was investigated in wild rodents [5]. Moreover, Lithuania is the only European country reporting a substantially growing human TBE incidence rate (from 12.5 cases per 100,000 population in 2012 to 24.3 in 2020). The incidence of TBE in other European countries has been stable for a decade or even declined. The incidence rate of TBE has declined in neighboring Latvia and Estonia (from 11.2 and 13.4 cases per 100,000 population in 2012 to 9.4 and 5.2 cases in 2020, respectively) [6]. Furthermore, suspensions and isolates in cell cultures of the brain and internal organ mix of each rodent were analyzed separately by PCR. In addition, a greater diversity of internal organ tissues was taken for RNA extraction, compared to some other studies.

The Lithuanian landscape rich in agricultural fields with hedgerows, woodlands, and forests, accompanied by humid climatic conditions is highly convenient for rodents – the main reservoir hosts of TBEV, and also ticks, the vector of the virus [386, 387]. Rodents and other small vertebrates are natural inhabitants of hedgerows, notably *C. glareolus* and *Apodemus* spp.

[127], which are common hosts of *I. ricinus* larvae and nymphs [158, 388] and were the most abundant captured rodent species in the present study. A study by Labuda et al. [389] suggested that *A. flavicollis* mice might be the most adapted species to TBEV and *I. ricinus* ticks and are highly suitable for virus transmission during co-feeding. Moreover, *A. flavicollis* mice show a significantly higher transmission rate than the bank vole (*C. glareolus*), the second most common rodent species in European forests. The agricultural landscape is an important factor for the abundance and species richness of *M. arvalis*, *A. agrarius*, and *A. sylvaticus* rodents [390], which were also collected in the present study, however, rodents of these species were captured in lower numbers.

Furthermore, the relative abundance of competent hosts (small mammals) to incompetent but tick-amplifying hosts (deer – the main host for nymph and adult feeding and adult tick mating) is an important driver of the number of nymphs feeding on rodents and hence suggested to be a key element in the persistence of TBEV [188, 391]. In addition, studies indicate that an abundance of deer might be related to a higher risk of TBE in humans [203, 392]. According to a recent investigation by Balčiauskas and Kawata [393], the population of deer in Lithuania has been exponentially increasing in the past two decades.

Forest fragmentation is also suggested to be related to higher TBEV prevalence [218]. A study conducted more than ten years ago concluded that forest fragmentation in Lithuania is so prevalent that it could potentially influence ecological processes in most forest landscapes [394]. However, the forest area in Lithuania increased by 6.2% from 2004 to 2019 following reforestation [386]. Moreover, TBEV was detected in Lithuania's green zones and city parks [395]. Furthermore, a retrospective epidemiological study conducted by Radzišauskienė and colleagues [341] found that 42% of TBE patients were infected in the living area and 60% were residents of cities. The results indicate a high TBE risk in Lithuania even in urban areas.

3.3.2. Comparison of TBE viral load in rodent tissue suspensions and isolates in Neuro-2a cells

A low TBE viral load was found in the major part of the brain and internal organ mix suspension samples of wild rodents. Overall, the amplification was observed only at threshold cycle (Ct) values of 35–40 in 70.1% of PCR-positive brain and internal organ mix suspension samples of rodents. To compare viral load before and after isolation in Neuro-2a cell culture, brain and internal organ mix suspension samples (n = 30) with the lowest Ct value and respective samples of isolates in neuroblastoma cells (n = 30) were taken

and TBEV viral load was measured. The average viral load in Neuro-2a cell culture isolates was found to be significantly higher ($p < 0.05$) compared to respective tissue suspension samples of the same rodents (6.35 $\log_{10}$ copies/mL and 5.71 $\log_{10}$ copies/mL, respectively) (Fig. 3.3.2.1). Moreover, successful virus isolation in murine neuroblastoma Neuro-2a cell culture confirmed viable TBEV presence in brain and internal organ mix samples of rodents.

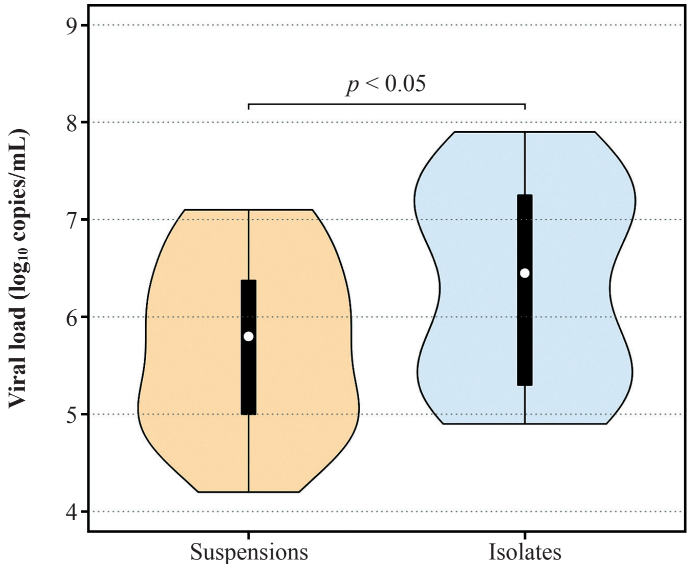

Fig. 3.3.2.1. *TBEV load in selected rodent brain and internal organ mix samples (n = 30) before (suspensions) and after (isolates) isolation in murine neuroblastoma Neuro-2a cells. The confidence intervals of the average viral load of all samples (vertical black line) and separate samples (width of the figures) are represented*

Although the cell culture method is time-consuming and requires considerable laboratory equipment and technical expertise, it has been regarded as the "gold standard" method for diagnosing viral disease for decades and remains a useful approach nowadays [396, 397]. As far as we know, the present study is the first successful attempt to isolate wild rodent-derived TBEV in murine neuroblastoma Neuro-2a cells to investigate the virus prevalence rate. A similar study was conducted by Kozuch et al. [141] with an unsuccessful attempt to isolate TBEV in chicken embryo cell culture from *A. flavicollis* blood samples. Experimental studies show that serial passaging in cell culture is necessary for virus adaptation to a specific cell line. Meanwhile, the structural rearrangements that favor viral load increase to detectable levels have time to develop [398]. Růžek et al. [399] have demonstrated that cell

lines of neural origin are highly susceptible to TBEV infection providing 100- to 10000-fold higher virus titer than cells of non-neural origin. Furthermore, successful virus detection in cell culture isolates from the brain and internal organ mix samples of rodents that were PCR-negative in suspension samples has demonstrated that murine neuroblastoma Neuro-2a cells are highly susceptible to rodent-derived TBEV infection. As a result, compatible viral host and cell line origin may have contributed to a high virus isolation success from naturally infected rodents revealed in the present study.

In line with the results of the present study, previous European studies have also reported low TBEV copy numbers in brain and spleen samples of small wild rodents collected in Germany [20, 371]. A viable TBEV from the brain and internal organ samples of experimentally infected bank vole that showed low Ct values (< 36.00) was successfully isolated in human lung carcinoma A549 cells by Michelitsch et al. [400]. The success of virus isolation was suggested to be related to different TBEV strains. The more recently isolated TBEV strains were more effectively reisolated in cell cultures as the reference Neudörfl strain, which indicates that virus strain plays a role in the success of virus isolation in cell culture [400]. In a more recent study, Michelitsch et al. [145] reported the successful isolation of TBEV in human lung carcinoma A549 cell culture from the whole blood samples of experimentally infected *C. glareolus* voles and concluded, that the recently isolated TBEV strains more frequently persisted as a long-term viremia and showed a higher detection rate of viral RNA in various organs, compared to the reference strain Neudörfl. Moreover, an experimental study on human blood infection with TBEV showed that the activation and suppression of the expression of adhesion and activation receptors were TBEV-induced and associated with the virus strain and thus, the different virulence and pathogenicity of the strains [401]. Interestingly, Bakker et al. [402] have successfully isolated TBEV from 7 out of 29 experimentally infected wild mice (mainly *A. flavicollis*), but only from male rodent brains with roughly $\geq 5 \times 10^3$ copies/µg. The susceptibility and virus persistence in experimental infection differs from natural infection, as was demonstrated in experimentally and naturally infected *Clethrionomys rutilus* and *A. agrarius* [144].

These data suggest that due to low viral load in the main part of rodent samples, the TBEV RNA detection by PCR might be complicated and insufficient in reservoir hosts. Although PCR is considered a highly specific and sensitive method for viral genome detection, significant limitations in reliably detecting the TBE virus were revealed in a TBEV detection quality control study [403]. Human blood serum samples containing different strains and amounts of the TBEV and two other related flaviviruses were provided to

23 European laboratories. Surprisingly, only two laboratories correctly identified all samples in the qualitative study [403].

Several studies have demonstrated the latency of TBE infection, which might be related to low viral load detected in the main part of small wild rodents. TBEV was detected persisting in the brains of small wild rodents and insectivores of various species in several of Europe's and Asia's investigations [144, 371, 372, 400, 404]. A natural TBE infection was demonstrated to persist for up to 10 months in *C. rutilus* voles [405]. It was suggested that in endemic regions TBEV persists in the major part of susceptible wild rodents and insectivores. However, a weak virus-specific immune response might enhance virus penetration in host monocytes and possibly result in difficulty in recovering pathogenic TBEV from its natural hosts [404, 406]. Moreover, it is hypothesized that certain genetic features and transcriptome profiles of the candidate rodent reservoir host predispose to asymptomatic carriage of TBEV and consequent transmission to *I. ricinus* ticks [380].

3.3.3. The influence of air temperature on TBEV prevalence in samples of rodent brain and internal organs

A successful TBE virus isolation in murine neuroblastoma cell culture from rodent samples (n = 30) that were PCR-negative before cultivation was demonstrated in the present study. The prevalence rate of TBEV RNA in samples of rodent brain and internal organ mix isolates in murine neuroblastoma Neuro-2a cell culture from all collection sites was analyzed when grouped according to the month of trapping and therefore, according to the average monthly air temperature (Supplementary Material Table S1). A strong correlation ($r = 0.88$; $p < 0.001$) was found between the average monthly air temperature of rodent collection and TBEV RNA prevalence rate in murine neuroblastoma cell culture isolates of rodent brain and internal organ mix samples which were PCR-negative before cultivation in Neuro-2a cells (Fig. 3.3.3.1).

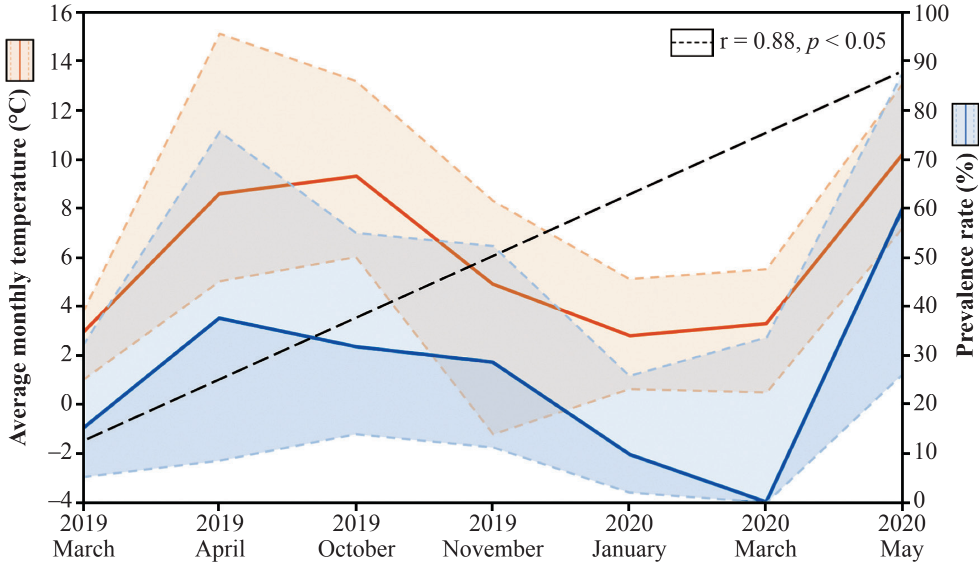

Fig. 3.3.3.1. *Longitudinal distribution of rodent brain and internal organ mix suspension samples considered negative prior isolation in Neuro-2a cell line and an average air temperature of the rodent trapping month. The outlined shades of different colors visualize confidence intervals*

The results show that TBEV RNA was more often detected in rodents captured at a lower average air temperature than those collected at a higher average temperature. The results of the present study suggest that when the average air temperature of rodent trapping increased, it led to a lower RNA detection rate in suspensions and an increased detection rate in cell culture isolates of the same rodent samples.

As far as we know, the present study is the first to suggest such a tendency in wild rodents. However, a higher TBEV-Eu RNA prevalence rate in a colder winter (54.2%) compared to a warmer one (5.6%) was reported in Finland. In the abovementioned study, the prevalence of TBEV RNA was analyzed two years in a row and the trapping area, month, and species of rodents were the same in both years [372]. Moreover, the influence of air temperature on TBEV replication in mammals was demonstrated in Northern white-breasted hedgehogs (*Erinaceus roumanicus*). Based on the results of an experimental study conducted by Nosek and Grulich [407] the duration of viremia in hedgehogs is indirectly related to air temperature, lasting 3–6 days in warm summer and 8–14 days in cooler spring and autumn. Furthermore, the persistence of intense and long-lasting viremia throughout the hibernation period in infected Northern white-breasted hedgehogs and dormice (*Glis glis*) was observed in the abovementioned study [407].

In contrast, the opposite trend was suggested in ticks, and a higher air temperature was associated with increased human TBE incidence rate and potentially higher TBEV load in ticks [11, 14]. Similarly, Korenberg and Kovalevskii [408] have also suggested that the incidence of clinical human TBE cases most closely corresponds to the real probability of being bitten by a highly TBEV-infected tick. Moreover, Hudáčková and colleagues [409] reported that the majority of human TBE cases in Czechia during 2012–2016 were recorded when the air temperature was 10–20 °C. Furthermore, Elväng et al. [410] proposed a thermosensitive RNA switch as significant for TBEV virus propagation in ticks.

Nonetheless, many other factors, such as relative humidity, duration of day length [11, 380], reproductive state and immune status [411], and tick burden on rodents [412] might be influential since these factors are at least partially related to the average air temperature. Moreover, the age and sex of rodents might also play a role [373]. A higher seroprevalence of TBEV-specific antibodies detected in male bank voles (40.4%) compared to females (15.6%) was associated with an increased risk of possible contact with ticks due to larger territories and higher activity in search of females [373, 377, 411]. Moreover, sexually active males might exhibit reduced innate and acquired immunity due to high testosterone levels [164, 413].

Various factors, such as stress, reproduction status, food availability, and climatic conditions might impact a host's immune system response and therefore, the virus replication in the rodent organism. Early spring could be a favorable time for TBEV activation from a latent state in rodent organisms due to a weakened immune response during winter [414]. Inhibition of male rodent humoral immunity is associated with increased testosterone levels in early spring when mating season begins. Activation of latent TBEV infection was demonstrated in an experimental study after injection of testosterone into male Northern red-backed voles (*C. rutilus*) [404]. Furthermore, TBEV activation was shown in experimentally infected Syrian hamsters (*Mesocricetus auratus*) given immunosuppressive drugs, such as vincristine, prednisolone, and adrenaline [415].

In addition, several studies have demonstrated that TBEV is heterogeneous, and both – adapted to tick and mammal cell variants are present at different quantities in the same host. The TBEV quasispecies existence was demonstrated in tick-derived TBEV samples [416−419]. Although the exact mechanism of quasispecies replication during natural infection remains unclear, virus replication in the organism of rodents might be influenced by host immune response, tick infestation, and other yet unknown factors that might induce the emergence of different virus variants [414, 416].

3.3.4. The prevalence of TBEV in different species of rodents

Many epidemiological and experimental studies conducted in Europe and Asia reported rodents of the *Apodemus* and *Clethrionomys* as one of the most important in the TBEV transmission cycle [420]. Mice of the genus *Apodemus* are the most widespread small rodents in natural habitats such as broadleaf forests and fields, in the Temperate Zone of the Palearctic region, and more than twenty species are now recognized [421]. The genus *Clethrionomys* comprises several species that have a global distribution and *C. glareolus* is a common rodent in Europe and North Asia. The distribution of *C. glareolus* overlaps with regions in which TBEV is endemic [22, 422]. Although the prevalence of different rodent species depends on numerous factors in various areas, the most abundant species in TBEV in European studies in rodents are *A. flavicollis* and *C. glareolus* (reviewed below).

Rodents of 6 species were trapped and investigated in the present study (Fig. 3.3.4.1) The most abundant of all collected rodents were *A. flavicollis* (n = 76), followed by *C. glareolus* (n = 29), *M. arvalis* (n = 22), and less numerous *M. musculus* (n = 9) *A. sylvaticus* (n = 2) and *A. agrarius* (n = 1).

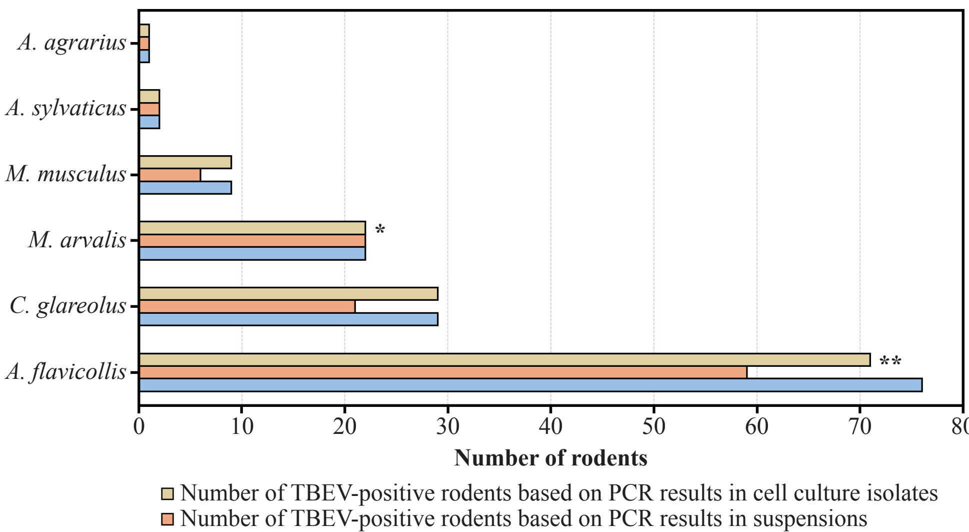

Fig. 3.3.4.1. *Number of TBEV-positive rodents of different species based on PCR results in suspensions and cell culture isolates.*
$* p < 0.05$; $** p = 0.46$ (Fisher's exact test)

TBEV RNA was found in the brain and/or internal organ mix suspensions of all (100%) *M. arvalis* rodents investigated in the present study. However, common voles were not abundant in the TBEV prevalence studies in rodents. The prevalence rate of 11% in *M. arvalis* rodents collected in a non-risk area in Germany [371] and 6.2% in a high-risk area in Hungary were previously reported [370]. Moreover, persistent TBEV infection for up to 100 d.p.i. was demonstrated in experimentally infected *M. arvalis* rodents [371]. TBEV-specific antibodies were detected in 3.3% of *M. arvalis* captured in Slovakia (although only 5.5% of all collected rodents were *M. arvalis*) [375]. In addition, a study conducted in Czechia revealed that the abundance of *M. arvalis* voles was a significant factor in explaining the annual human morbidity of TBE and two other zoonoses [423].

The prevalence rate of TBEV RNA in brain and internal organ mix suspension samples was significantly higher ($p < 0.05$) in *M. arvalis* rodents, compared to other species included in the present study. In addition, the majority (77.3%) of *M. arvalis* rodents were collected in January and March of 2020 when the average air temperature was only 2.8–3.3 °C and ticks were not yet at the peak of their activity. It is important to note that the warmest January from 1961, was registered in 2020 and might have influenced the results of TBEV prevalence in the rodents collected that month. However, it remains unclear if the season and average air temperature at the time of rodent collection or the rodent species were the influential factors for the higher prevalence in suspension samples of *M. arvalis* before cultivation in murine neuroblastoma cell culture.

The TBEV RNA was detected in 77.6% (59/76) of *A. flavicollis* brain and/or internal organ mix suspension samples investigated in the present study. *A. flavicollis* mice are considered one of the most abundant species in Europe, however, only a few studies have evaluated TBE viral RNA prevalence in these rodents. In Germany, TBEV RNA was detected in 13% of *A. flavicollis* captured in the TBEV risk area and 2% of rodents in the non-risk area [371]. In TBEV-risk areas in Hungary, TBEV RNA was found in 4% of yellow-necked mice samples [370]. The reported seroprevalence of TBEV-specific antibodies in *A. flavicollis* was the highest and reached 18.1% in Slovakia [375], followed by 10.5% in Germany [373], 3.9% in Slovenia [22], and 3.7% in Hungary [377].

The prevalence of TBEV RNA in brain and internal organ mix suspension samples of *A. flavicollis* mice was significantly lower (Fisher's exact test $p = 0.46$) compared to other species of rodents investigated in the present study. However, after cultivation in murine neuroblastoma Neuro-2a cell culture, no significant differences between rodent species and the prevalence rate of TBEV RNA were found. The overall TBEV RNA prevalence rate

increased significantly to 96.4% (95% CI 91.8–98) following each rodent sample cultivation in murine neuroblastoma cell culture. TBEV RNA was absent in only 5 of 139 rodents and all of them were rodents of *A. flavicollis* species (Supplementary Material Table S2). The results of the present study suggest that TBEV is not more prone to be found in specific rodent species. This finding is in line with the results of Achazi and colleagues [371] who also did not find significant TBEV RNA prevalence rate differences between the species of rodents. Moreover, Bakker et al. [402] found no significant difference in infection rates between *A. sylvaticus* and *A. flavicollis* mice in an experimental study. Furthermore, an experiment by Heigl showed that *A. flavicollis*, *C. glareolus, A. sylvaticus,* and *M. musculus* were susceptible to TBEV infection [424].

TBEV RNA was detected in 72.4% (21/29) of *C. glareolus* brain and/or internal organ mix suspension samples in the present study. Studies reporting the prevalence of TBEV in *C. glareolus* are more abundant than those reporting TBEV presence in *A. flavicollis*. TBEV-Sib RNA was detected in 11.4% of *C. glareolus* trapped in high-risk areas in Finland [372]. TBEV-Eu RNA was found in 11% of bank voles trapped in non-risk locations, 18% trapped in high-risk locations in Germany [371], and 4% in high-risk areas in Hungary [370]. An experimental study by Tonteri et al. [425] demonstrated that *C. glareolus* rodents are susceptible to three main subtypes of TBEV – TBEV-Eu, TBEV-Sib, and TBEV-FE. In addition, the same study showed that viral RNA can be detected at 109 d.p.i. in the brain of experimentally infected *C. glareolus*, however, the authors did not attempt to isolate the virus [425]. In a study conducted by Stoltz et al. [426] primary cells obtained from *C. glareolus* fetuses were successfully infected with TBEV derived from human blood serum and the presence of the virus was demonstrated by immunofluorescent staining of viral proteins. TBEV was isolated in cell culture and propagated in mouse brains from the brain sample of *C. glareolus* captured in Slovakia [427]. In addition, TBEV was isolated from the lungs, kidneys, and spleen of *C. glareolus* trapped in Czechia [428]. In a study conducted in Slovenia by Knap et al. [22] a very high average TBE viral load (2.5×10^9 RNA copies/mL) was reported per organ in *C. glareolus*, however, the levels varied in different tissues. In the abovementioned study, TBEV RNA was detectable in various tissue samples of *C. glareolus* such as the spleen, kidney, lung, liver, heart, blood clots, and brain [22]. The prevalence rate of TBEV-specific antibodies in *C. glareolus* reaching 20.5% was reported in Hungary [377], 19.4% in Germany [373], 15.1% in Slovakia [375], 12.5% in Slovenia [22], and 5.3% in Switzerland [379]. Other species of the genus *Clethrionomys* were also reported to be highly susceptible to TBEV infection. A study conducted by Bakhvalova et al. in Siberia reported that TBEV RNA

prevalence was 46.2% in *Clethrionomys rufocanus* and 78.1% in *C. rutilus* [369].

In the present study, TBEV RNA was detected in 66.7% (6/9) of brain and/or internal organ mix samples of *M. musculus* house mice. The data on the prevalence of TBEV in *M. musculus* rodents is scarce, possibly because of the low numbers of these species collected in TBEV epidemiological studies. *M. musculus* mice were mentioned only in one study where no TBEV RNA was detected mainly because of the low number of samples (n = 2) [371]. However, experimental studies show that *M. musculus* rodents are susceptible to TBEV infection [424, 429]. As far as we know, the present study is the first to report TBEV RNA detection in naturally infected house mice *M. musculus*.

TBEV RNA was prevalent in brain and/or internal organ mix suspension samples of 100% of *A. sylvaticus* and *A. agrarius* in the present study. However, only two rodents of *A. sylvaticus* and one of *A. agrarius* species were collected, therefore the results of TBEV prevalence in these species are not accurate due to a low number of samples. Although both species mentioned above were found to be sufficient TBEV hosts in other studies.

A prevalence of TBEV RNA was detected in 11% of *A. agrarius* collected in a non-risk area in Germany [371] and 7.3% in a high-risk area in Hungary [370]. Moreover, TBEV was isolated in cell culture from the lungs and spleen tissue of *A. agrarius* rodents collected in the Republic of Korea [70, 430]. In addition, TBEV-FE was isolated from *A. agrarius* in Estonia [69]. The prevalence rate of TBEV-specific antibodies in 4.6% of *A. agrarius* collected in Hungary [377] and 2.4% trapped in Slovenia were reported [22]. Moreover, Egyed et al. [431] demonstrated that experimentally infected *A. agrarius* may develop subclinical TBE encephalitis depending on virus load in the inoculum and that the rodents of the genus *Apodemus* might be resistant to lower infectious dose.

An experimental study evaluated the immune response to TBEV infection in *A. sylvaticus* rodents [432]. Bakker et al. [402] suggested that in the Netherlands *A. sylvaticus* and *A. flavicollis* may equally contribute to the TBEV transmission cycle. TBEV-specific antibodies were detected in 9.6% of *A. sylvaticus* captured in Slovenia [22], 8.5% in Slovakia [375], and 2.9% in Switzerland [379].

A large-scale epizootiological study conducted in Japan reported a prevalence rate of TBEV-specific antibodies in 5.51% of the voles of genus *Clethrionomys* and 1.81% of *Apodemus* mice [433]. Moreover, infestation with ticks was reported to be higher in *A. flavicollis* compared with *C. glareolus* in several studies [169, 377], possibly because of the development of resistance to ticks observed in *C. glareolus* rodents [23, 162]. Furthermore, several

studies have demonstrated that *C. glareolus* rodents might be more susceptible to TBEV infection compared to *A. flavicollis*, however, it has been implied that the rate of infection in a specific species may also depend on the year of investigation [22, 375, 428]. In several experimental studies, *C. glareolus* was demonstrated to produce higher viremia and higher antibody titers in comparison to the rodents of the *Apodemus* species [375, 389, 424, 429]. Moreover, a study by Knap et al. [22] suggested that the virus tropism of TBEV in *Apodemus* mice is different from that in *Clethrionomys* species since the virus was predominantly detected in the spleen and less often in the brain, lungs, and blood clots. In addition, viral loads in *Apodemus* rodents were generally lower compared to *Clethrionomys* species. Furthermore, although the *Apodemus* mice seroconvert following exposure to TBEV, the antibody titers were lower than in *Clethrionomys* voles [22]. The presence of viral RNA in multiple tissues indicates that TBEV infection in the *Clethrionomys* species is not confined to a single organ, and high viremia levels suggest that *Clethrionomys* voles may transmit the virus to naïve ticks that feed on them [420].

The data of multiple studies indicate that *C. glareolus* rodents might be more suitable hosts for systemic transmission of TBEV to ticks, however, it is important to note that the viremia in rodents might be short-term in many cases [22, 140, 143, 371, 424]. On the other hand, experimental studies have suggested *A. flavicollis* as a more competent rodent species compared to *C. glareolus* for the TBEV transmission to naïve ticks during tick co-feeding on the non-viremic host [101, 130]. An experimental study conducted by Labuda et al. [130] demonstrated that field mice (*A. flavicollis* and *A. agrarius*) maintained virus transmission to a higher proportion of ticks (to 46% of exposed ticks) when carried comparatively low or even undetectable viral load compared to *C. glareolus* voles (to 28% of exposed ticks) which showed higher viremia. In conclusion, the reported data suggests that rodents of both genera are important in the maintenance cycle of TBEV.

3.3.5. The prevalence of TBEV in fetuses of small wild rodents

Five fetal samples were investigated in the present study (which were not included in the overall rodent count). TBEV RNA was detected in 2 out of 5 fetus suspension samples. After cultivation in murine neuroblastoma Neuro-2a cell culture, all 5 fetal samples were TBEV-positive. In *M. arvalis* voles, TBEV RNA was detected in maternal and fetal samples before and after cultivation in murine neuroblastoma cell culture. In *C. glareolus* voles, TBEV RNA was detected only in maternal suspension samples before cultivation in neuroblastoma cells. In one of three *A. flavicollis* mice, TBEV RNA

was found in the fetal suspension sample but not in the maternal suspension samples before sample cultivation in cell culture. In the remaining two *A. flavicollis*, TBEV RNA in maternal and fetal samples were detected only after cultivation in murine neuroblastoma Neuro-2a cell culture.

As far as we know, no previous studies have found TBEV RNA in the fetuses of *A. flavicollis*, *M. glareolus,* and *M. arvalis* rodents. However, in two experimental studies vertical TBEV-Sib transmission was demonstrated in infected laboratory mice and *C. rutilus* females [434, 435]. In the investigation by Bakhvalova et al. [434] vertical virus transmission was reported in up to 90% of experimentally infected Northern red-backed vole (*C. rutilus*) progeny. Moreover, transplacental TBEV transfer to embryos was observed in naturally infected *C. rutilus* in the abovementioned study [434]. The first case of possible intrauterine vertical TBEV transmission in Dalmatian puppies was recently reported. All three infected puppies developed severe neurological symptoms and died [315]. Vertical TBEV transmission in humans was revealed in a few studies [238, 269, 436]. To date, no cases of harmful effects of TBEV vertically transmitted to human newborns have been reported. Although other flaviviruses might cause harm to embryos in case of transplacental transfer: the Zika virus is teratogenic, and the Dengue virus and Japanese encephalitis virus are associated with an increased risk of fetal loss [437]. Furthermore, sexual TBEV transmission was reported between infected males and uninfected females in laboratory mice, which are not adapted TBEV hosts [438].

CONCLUSIONS

1. TBEV RNA was detected in 18.6% (95% CI 15.2–22.4) and TBEV-specific antibodies were found in 21.6% (95% CI 17.9–25.6) of blood serum samples of dogs.
2. The risk factors of the lethal outcome of the disease for TBEV RNA-positive dogs were: the older age of the dog (on average 11.8 years; $p < 0.01$), the presence of neurological symptoms ($p < 0.01$), and the presence of TBEV-specific antibodies in blood serum ($p < 0.05$) in PCR-positive dogs with severe neurological symptoms.
3. The detection rate of TBEV RNA in ticks, collected from dogs increased significantly ($p = 0.01$) after sample cultivation in MARC-145, Vero, and Neuro-2a cell cultures from 34.2% (95% CI 25.7–43.5) in suspensions of ticks to 56.4–60.7% (95% CI 46.9–69.6) in cell culture isolates.
4. TBEV RNA was detected in 51.1% (95% CI 42.4–59.7) of the rodents' brains and 53.2% (95% CI 44.6–61.7) of the internal organ mix suspensions. TBEV RNA was significantly more often found in suspensions of *M. arvalis* rodents and less often in *A. flavicollis* rodents than other species.
5. The detection rate of TBEV RNA in rodents increased significantly ($p < 0.01$) after sample cultivation in Neuro-2a cell cultures from 74.8% (95% CI 66.7–81.1) in brain and internal organ mix suspensions to 96.4% (95% CI 91.8–98) in Neuro-2a cell culture isolates.
6. A strong correlation ($r = 0.88$; $p < 0.05$) was found between the average monthly air temperature of rodent trapping and TBEV RNA prevalence rate in cell culture isolates of rodent brain and internal organ mix samples which were PCR-negative before cultivation in murine neuroblastoma cells.

SUMMARY IN LITHUANIAN

1. PROBLEMOS AKTUALUMAS IR SVARBA

Erkinis encefalitas (EE) yra viena svarbiausių erkių platinamų zoonozių Europoje ir Šiaurės Azijoje [1]. Ligą sukelia erkinio encefalito virusas, kuris priklauso *Flaviviridae* šeimai [2]. Dažniausiai užsikrečiama įsisiurbus virusu užkrėstai erkei, tačiau galimas ir alimentinis užsikrėtimas per nepasterizuotus virusu užkrėstus pieno produktus [3]. Medicinoje tai viena svarbiausių erkių platinamų infekcijų, galinčių sukelti sunkių neurologinių simptomų, liekamųjų reiškinių persirgus arba baigtis mirtimi [4].

Nors ligos galima išvengti pasiskiepijus, žmonių sergamumas pastaraisiais dešimtmečiais auga, todėl tai yra didėjanti sveikatos apsaugos sistemos problema beveik visose EE endeminėse šalyse tiek Europoje, tiek ir Azijoje [4]. Per pastaruosius 30 metų žmonių sergamumas EE endeminiuose Europos regionuose padidėjo beveik 400 proc. [5]. Taip pat fiksuojamas endeminių EE rizikos vietovių plėtimasis bei naujų židinių atsiradimas šalyse, kurios anksčiau buvo laikomos laisvos nuo EE. Europos Sąjungoje ir Europos ekonominėje erdvėje (ES/EEE) EE atvejų pranešimų skaičius pastaraisiais metais toliau didėja [5, 6]. Europos ligų prevencijos ir kontrolės centras (ECDC) praneša, kad 2016–2018 metais sergamumas EE siekė 0,6 atvejo 100 tūkst. gyventojų, 2019 metais – 0,7 atvejo 100 tūkst. gyventojų ir 2020 metais padidėjo iki 0,9 atvejo 100 tūkst. gyventojų [5]. Sergamumas EE Lietuvoje yra vienas didžiausių Europoje jau daugiau nei dešimtmetį. Nacionalinio visuomenės sveikatos centro duomenimis, 2023 metais Lietuvoje EE atvejų užfiksuota 58 proc. daugiau nei 2022 metais ir sergamumo rodiklis padidėjo nuo 13,3 iki 20,8 atvejo 100 tūkst. gyventojų.

Erkinis encefalitas yra gamtinė židininė infekcija, o viruso paplitimas gamtoje, kaip manoma, priklauso nuo erkių ir jų šeimininkų tarpusavio sąveikos bei aplinkos ir klimato sąlygų [7–10]. Klimato šiltėjimas yra laikomas vienu iš pagrindinių veiksnių, lemiančių didėjantį žmonių sergamumą EE [8, 11–14]. Didėjantis sergamumas taip pat siejamas ir su dažnėjančia žmonių veikla potencialiai erkėmis užkrėstose vietovėse bei tų vietovių plėtimasis [10, 15], ypač urbanizuotose teritorijose bei priemiesčiuose [16, 17]. Kadangi tarp erkių erkinio encefalito viruso (EEV) paplitimo dažnis yra nedidelis, ypač krauju nepasimaitinusių erkių [18], buvo tyrinėjami įvairių rūšių gyvūnai, kaip potencialūs indikatoriniai gyvūnai, EEV židiniams aptikti ir stebėti [19–21].

Smulkieji laukiniai graužikai yra pagrindinis gamtinis viruso šaltinis ir yra laikomi svarbiausiais rezervuariniais EEV šeimininkais, dėl to jie potencialiai itin tinkami EEV židinių stebėjimo tyrimams [20]. Smulkieji laukiniai

graužikai nemigruoja tolimų atstumų, jų populiacijos dažniausiai gana didelės, o graužikų gaudymas nesudėtingas [22]. Jie linkę gausiai užsikrėsti erkėmis ir yra labai svarbūs *Ixodes* erkių lervų ir nimfų šeimininkai [23, 24]. Didelio endemiškumo vietovėse Sibire atlikti tyrimai suponuoja, kad EEV židiniuose didžioji dalis smulkiųjų laukinių graužikų yra latentiniai EEV nešiotojai [25]. Nustatyta, jog EEV-Eu potipio virusas gali išlikti (persistuoti) graužikų organizme nesukeldamas imuninio atsako ir antikūnų gamybos [26, 27].

Erkinio encefalito virusu gali užsikrėsti įvairių klasių gyvūnai – reptilijos, žinduoliai, paukščiai, tačiau EE simptomai dažniausiai pasireiškia žmonėms, rečiau šunims ir arkliams ir retkarčiais kitiems atsitiktiniams viruso šeimininkams [28]. Remiantis pavienėmis publikacijomis ir klinikinių atvejų pranešimais, paskelbtais dar praėjusiame šimtmetyje, erkinio encefalito atvejų šunų populiacijoje buvo diagnozuojama itin retai. Manyta, kad pasireiškus klinikiniams požymiams ligos eiga būna sunki, o gaištamumas didelis [29]. Tačiau endeminėse vietovėse EEV dabar yra laikomas vienu svarbiausių CNS infekcijų sukėlėjų šunims [30]. Nors atliktų studijų duomenys rodo, kad užsikrėtimas EEV šunims dažniausiai yra asimptominis, tačiau taip pat užfiksuota ir žaibinės, ūminės bei lėtinės ligos eigos atvejų [21, 29, 32]. Manoma, kad net ir endeminiuose regionuose EE gali būti nediagnozuojamas šunims dėl nepakankamos informacijos apie šią ligą šunims [32–34]. Liga įtariama atsižvelgiant į būdingus klinikinius požymius, o diagnozė patvirtinama kraujo serume ir (arba) cerebrospinaliniame skystyje (CSS) aptikus EEV specifinių IgM klasės antikūnų [35, 36], tačiau galimas ir kryžminis kitiems flavivirusams specifinių antikūnų reaktyvumas [37]. EEV RNR aptikimas PGR metodu gali būti naudingas įrankis ankstyvajai EE diagnozei [36].

Šio darbo tikslas buvo ištirti EEV paplitimą viruso židiniuose sugautų graužikų ir šunų, gyvenančių antrajame pagal dydį Lietuvos mieste, organizmuose. Remiantis anksčiau atliktų studijų duomenimis apie viruso išlikimą (persistavimą) graužikų organizme, buvo iškelta hipotezė, jog mėginių pagausinimas pelių neuroblastomos „Neuro-2a“ ląstelių kultūroje galėtų padidinti viruso kiekį ir taip leistų jį aptikti. Be to, pirmą kartą Lietuvoje buvo atlikti EEV RNR ir EEV specifinių antikūnų tyrimai šunų klinikiniuose mėginiuose ir nustatyti galimi sunkesnės ligos eigos ir letalios baigties rizikos veiksniai.

2. MOKSLINIS NAUJUMAS

EEV paplitimas tarp smulkiųjų laukinių graužikų ir šunų Lietuvoje anksčiau nebuvo išsamiai tyrinėtas. Pirmą kartą Europoje viruso židiniuose

sugautų graužikų organizmuose buvo nustatytas didelis EEV paplitimas. Pirmą kartą Europoje EEV aptiktas smulkiųjų graužikų vaisiaus mėginiuose, anksčiau publikacijose nepaminėtose graužikų rūšyse (*A. flavicollis*, *C. glareolus* ir *M. arvalis*). Pirmą kartą natūrali EEV infekcija nustatyta naminių pelių (*M. musculus*) organizmuose.

Pirmą kartą Lietuvoje ištirtas EEV ir jam specifinių antikūnų paplitimas šunų klinikiniuose mėginiuose. Nustatyti rizikos veiksniai, galbūt lemiantys sunkesnę ligos eigą ar gaišimą. Surinkti duomenys aktualūs ir nauji pasauliniu mastu, taip pat svarbūs ir žmonių medicinai, nes suteikia daugiau informacijos apie viruso diagnostiką ankstyvosiose arba ūminėse ligos stadijose, kai EEV specifiniai antikūnai dar neaptinkami. EEV diagnostikos jautrumui įvertinti pirmą kartą sėkmingai panaudotas viruso pagausinimo ląstelių kultūroje metodas ir jo efektyvumas palygintas su standartiniais molekuliniais PGR tyrimų metodais.

3. DARBO TIKSLAS IR UŽDAVINIAI

Darbo tikslas: nustatyti erkinio encefalito viruso paplitimo dažnį tarp smulkiųjų laukinių graužikų ir šunų, taip pat nuo šunų nurinktų įsisiurbusių erkių.

Uždaviniai:

1. Nustatyti EEV ir EEV specifinių antikūnų paplitimo dažnį šunų kraujo mėginiuose ir įvertinti pagrindinius rizikos veiksnius, lemiančius sunkesnę šunų EE ligos eigą ir letalią baigtį.
2. Įvertinti EEV paplitimo dažnį nuo šunų nurinktų erkių suspensijose ir po pagausinimo persėjamosiose ląstelių kultūrose.
3. Nustatyti EEV paplitimo dažnį viruso židiniuose sugautų smulkiųjų laukinių *Muridae* ir *Cricetidae* šeimos graužikų smegenų ir vidaus organų mišinio mėginiuose.
4. Įvertinti EEV diagnostikos jautrumą atliekant smulkiųjų laukinių graužikų mėginių pirminį pagausinimą persėjamosiose ląstelių kultūrose ir taikant standartinius molekulinius tyrimų metodus.

4. TYRIMŲ METODAI IR TIRIAMOJI MEDŽIAGA

4.1. Mėginių rinkimas

4.1.1. Šunų klinikiniai mėginiai

Šunų kraujo mėginiai buvo renkami atsitiktine tvarka iš dviejų Kauno mieste esančių veterinarijos klinikų. Šunų klinikiniai mėginiai ir jų gyvenimo bei ligos anamnezė buvo surinkti gavus rašytinį gyvūno savininko ar globėjo

sutikimą. Šunų kraujo ir CSS mėginiai buvo imami gydymo ir diagnostikos tikslais, virusologiniams tyrimams papildomai mėginiai nebuvo imami. Visos procedūros ir tyrimai buvo atliekami laikantis gyvūnų gerovės reikalavimų bei Lietuvos Respublikos įstatymų.

Per laikotarpį nuo 2020 metų gegužės iki 2021 metų gruodžio buvo surinkti 473 šunų, atvykusių į veterinarijos klinikas dėl įvairių ligų ar profilaktinių sveikatos tyrimų, kraujo bei trys cerebrospinalinio skysčio mėginiai. Surinkti mėginiai buvo laikomi −80 °C temperatūroje, kol bus atlikti tolesni tyrimai.

Taip pat veterinarijos klinikose buvo diagnozuotos gretutinės kitos erkių platinamos infekcijos (*Babesia* spp. ir *Anaplasma phagocytophilum*), kurios buvo patvirtintos aptikus minėtus sukėlėjus rutininiu greituoju būdu dažytame kraujo tepinėlyje. Antikūnai prieš *A. phagocytophilum* buvo aptikti naudojant imunochromatografijos principu paremtą greitąjį testą (Anigen Rapid CaniV-4 Test Kit (BioNote, INC. Hwaseong, Korėjos Respublika).

Šunų *anamnesis vitae* ir *anamnesis morbi* duomenys bei ligos baigtis buvo surinkti retrospektyviai ir yra pateikti papildomoje lentelėje (Supplementary material Table S3).

4.1.2. Erkių mėginiai

Nuo šunų buvo nurinkta 117 prisisiurbusių erkių, iš jų 112 *I. ricinus* ir 5 *D. reticulatus*. Erkės buvo renkamos vizito veterinarijos klinikose metu. Surinkti mėginiai buvo laikomi −80 °C temperatūroje, kol bus atlikta tolesnė analizė.

4.1.3. Smulkiųjų laukinių graužikų mėginiai

Smulkieji laukiniai graužikai (*Rodentia*) buvo sugauti 19-oje skirtingų EEV židinių, esančių penkiose Lietuvos apskrityse. Specifinės graužikų gaudymo vietos buvo pasirinktos atsižvelgiant į Lietuvoje atliktos EEV paplitimo tarp erkių studijos duomenis didžiojoje dalyje pasirinktų židinių [320] bei į Nacionalinio visuomenės sveikatos centro pateiktą erkių platinamų ligų paplitimo Lietuvoje žemėlapį (*https://nvsc.lrv.lt/lt/uzkreciamuju-ligu-valdymas/uzkreciamosios-ligos/erkiu-pernesamos-ligos/lietuvos-vietoviu-kuriose-uzsikreciama-erkiu-platinamomis-ligomis-zemelapis/*). Gaudymui buvo naudojami gyvagaudžiai spąstai su saulėgrąžų aliejuje išmirkytos duonos masalu, o sugauti graužikai pristatyti į laboratoriją tą pačią dieną, kur vadovaujantis standartiniais protokolais buvo atliktas jų skrodimas ir paimtos smegenys bei vidaus organai (blužnis, kepenys, širdis ir inkstai) ir vaisiaus / embriono mėginiai iš penkių patelių. Paimti mėginiai buvo laikomi –80 °C temperatūroje, kol bus atliekami tolesni tyrimai. Iš viso nuo 2019 metų kovo

iki 2020 metų gegužės buvo sugauti 139 graužikai. Identifikuotos šešios graužikų rūšys: geltonkaklė pelė (*Apodemus flavicollis* (n = 76)), rudasis pelėnas (*Clethrionomys glareolus* (n = 29)), paprastasis pelėnas (*Microtus arvalis* (n = 22)), naminė pelė (*Mus musculus* (n = 9)), miškinė pelė (*Apodemus sylvaticus* (n = 2)) ir dirvinė pelė (*Apodemus agrarius* (n = 1)).

Vidutinės mėnesio oro temperatūros duomenys buvo paimti iš Lietuvos hidrometeorologijos tarnybos prie Aplinkos ministerijos pateiktų metinių ataskaitų (*https://www.meteo.lt/category/menesio-hidrometeorologiniu-salygu-apzvalga/page/5/).*

4.2. EEV RNR nustatymas ir sekoskaita

Smulkiųjų laukinių graužikų smegenų ir vidaus organų gabalėliai (10–100 mg) buvo homogenizuoti grūstuvėje su piestele iš kiekvieno graužiko organų sudarant du mėginius (smegenų ir vidaus organų mišinio), o erkių mėginiai prieš homogenizavimą buvo apdoroti skystuoju azotu. Paruošti mėginiai buvo sumaišyti su 1000 µl ląstelių kultūrų mitybinės terpės (MEM, Gibco, Waltham, MA, JAV) ir gautos suspensijos buvo laikomos –80 °C temperatūroje, kol bus atlikti tolesni tyrimai. Šunų kraujo ir CSS mėginiai RNR išgryninti buvo naudojami be papildomo paruošimo.

Siekiant iš mėginių išgryninti RNR, paimta 300 µl paruoštų graužikų smegenų ir vidaus organų mišinio suspensijų, erkių suspensijų bei šunų kraujo ir CSS, o RNR išgryninta pagal gamintojo instrukcijas naudojant komercinį RNR išgryninimo rinkinį „GeneJET RNA Purification Kit“ (Thermo Scientific, Waltham, MA, JAV).

Siekiant nustatyti EEV RNR, iš smulkiųjų laukinių graužikų smegenų ir vidaus organų mišinio suspensijų išgryninti RNR mėginiai buvo ištirti tikralaikės atvirkštinės transkripcijos polimerazinės grandininės reakcijos (AT-PGR) bei lizdinės AT-PGR metodais. Tikralaikės AT-PGR tyrimui naudoti „SuperScript™ III One-Step RT-PCR System“ reakcijos mišinys, „Platinum™ Taq DNA Polymerase“ polimerazė (Thermo Scientific, Waltham, MA, JAV) ir pradmenys, kaip nurodyta ankstesnėje studijoje [317]. Šunų kraujo ir CSS, erkių suspensijų bei izoliatų ląstelių kultūrose mėginiai buvo ištirti lizdinės AT-PGR metodu naudojant „DreamTaq Green PCR Master Mix“ reakcijos mišinį ir pradmenis, kaip nurodyta ankstesnėje studijoje [318]. Aptikto EEV fragmento specifiškumas buvo patvirtintas atlikus viruso genomo NCR fragmento sekoskaitą.

4.3. EEV specifinių antikūnų aptikimas

Specifiniams antikūnams prieš erkinio encefalito virusą nustatyti buvo naudojami komerciniai kiekybinės imunofermentinės analizės rinkiniai (ELISA) (VetLine TBE/FSME ELISA, NovaTec Immundiagnostica GmbH, Dietzenbach, Vokietija) pagal gamintojo nurodymus.

Siekiant patvirtinti aptiktų antikūnų specifiškumą EEV šunų kraujo serumo mėginiuose, kurie buvo teigiami arba abejotini, remiantis ELISA tyrimu, atliktas virusų neutralizacijos testas (VNT). Prieš atliekant tyrimą, šunų kraujo serumo mėginiuose inaktyvuoti komplemento baltymai 30 min. šildant mėginius vandens vonelėje 56 °C temperatūroje. Paruošti mėginiai buvo atskiesti ląstelių kultūrų mitybinėje terpėje (MEM) koncentracijomis nuo 1:5 iki 1:320. Atskiesti serumai buvo 90 min. inkubuojami su 100 $TCID_{50}$ EEV „Neudörfl" padermės (kuri buvo gauta po 6 serijinių persėjimų „Vero" (ATCC® CCL-81™) ląstelių kultūroje) 37 °C temperatūroje. Viruso citopatinis poveikis ląstelėms buvo vertinamas praėjus 3, 5 ir 7 dienoms po užkrėtimo. Didžiausias serumo atskiedimas, kuris slopino EE viruso citopatinį poveikį, buvo nustatytas galutinio taško titru. Serumai, kurių titras 1:20 ir didesnis, buvo laikomi teigiamais EEV specifinių antikūnų atžvilgiu.

4.4. Virusų išskyrimas ląstelių kultūrose

Paruošti smulkiųjų laukinių graužikų smegenų ir vidaus organų suspensijų, erkių suspensijų bei šunų kraujo ir CSS mėginiai buvo 10 min. centrifuguojami 12 000 × g greičiu ir nufiltruoti naudojant 0,22 µm porų dydžio mikrofiltrą (Techno Plastic Products AG, Trasadingen, Šveicarija). Pelių neuroblastomos ląstelės („Neuro-2a" ATCC Nr. CCL-131) buvo 90 min. inkubuojamos su paruoštu mėginiu plastikinėse ląstelių kultivavimo plokštelėse (TPP Techno Plastic Products AG, Šveicarija) 37 °C temperatūroje, po to nuplautos su 1x fosfatiniu buferiniu tirpalu. Ląstelės kultivuotos 200 µl ląstelių kultūrų mitybinės terpės (Dulbecco's Modified Eagle's Medium (DMEM, Gibco, JAV)), papildytos 10 proc. karščiu inaktyvuoto galvijų vaisiaus kraujo serumo (Gibco, JAV) bei 100 U/ml penicilino ir 100 µg/ml streptomicino ir inkubuotos 37 °C temperatūroje 5 proc. CO_2 aplinkoje.

Paruošti erkių suspensijų bei šunų kraujo ir CSS mėginiai buvo panaudoti beždžionių inkstų „Vero" (ATCC Nr. CCL-81) ir „MARC-145" (ATCC Nr. CRL-12231) ląstelių kultūrų užkrėtimui. Ląstelių užkrėtimas, inkubavimo ir kultivavimo sąlygos tokios pat, kaip aprašyta anksčiau, išskyrus tai, kad DMEM ląstelių kultūrų terpė buvo pakeista į MEM (MEM, Gibco, JAV).

„Vero" ir „MARC-145" ląstelės buvo kultivuotos 7 dienas, o „Neuro-2a" ląstelės – 4 dienas. Ląstelių užkrėtimas buvo atliekamas trigubais egzemplioriais, taip pat įtrauktos teigiamoji ir neigiamoji kontrolinės grupės. Po trijų

serijinių persėjimų ląstelės buvo užšaldytos −80 °C temperatūroje ir atšildytos du kartus. Ląstelių kultūrų suspensijos surinktos RNR ekstrakcijai. Sėkmingas viruso išskyrimas ląstelių kultūroje buvo patvirtintas tikralaikės AT-PGR ir lizdinės AT-PGR metodais.

4.5. Virusų kopijų kiekio nustatymas

Virusų kopijų skaičius buvo nustatytas pasitelkus modifikuotą metodiką, aprašytą ankstesnėse studijose [317, 319]. EEV kopijų skaičius buvo įvertintas pritaikius NS5 fragmento klonavimo metodiką. Į pJET 1.2 plazmidę buvo įterptas NS5 fragmentas naudojant komercinį klonavimo rinkinį „CloneJET PCR Cloning Kit“ (Thermo Scientific, Waltham, MA, JAV) pagal gamintojo nurodymus. Klonuotas NS5 fragmentas transformuotas į paruoštas *Escherichia coli* bakterijas naudojant „Transform-Aid Bacterial Transformation Kit“ (Thermo Scientific, Waltham, MA, JAV) pagal gamintojo instrukciją.

Plazmidžių DNR išgryninta naudojant komercinį rinkinį „GeneJET Plasmid Miniprep Kit“ (Thermo Scientific, Waltham, MA, JAV) pagal gamintojo instrukciją. Gautos DNR koncentracija įvertinta naudojant rinkinį „Qubit dsDNA BR Assay Kit“ (Invitrogen, Waltham, MA, JAV) pagal gamintojo nurodymus. Standartinė kreivė buvo paruošta pasitelkus dešimtkartinius plazmidžių DNR praskiedimus, o sugretinus juos su tikralaikės AT-PGR Ct vertėmis buvo gautas virusų kopijų ekvivalentų diapazonas nuo 10^2 iki 10^8. Viruso kopijų skaičius buvo apskaičiuotas pagal formulę:

$$n = \frac{x \cdot 6{,}0221 \cdot 10^{23}}{(N \cdot 660) \cdot 1 \cdot 10^{9}},$$

kur: n – virusų kopijų ekvivalento skaičius, x – amplikonų kiekis, N – dsDNR amplikono ilgis.

Visi mėginiai buvo tirti triplikatais ir apskaičiuotas matematinis vidurkis.

4.6. Statistinė analizė

Rizikos veiksnių reikšmingumui patikrinti buvo naudojama dvejetainė logistinės regresijos analizė, chi kvadrato testas ir Fišerio tiksliojo kriterijaus testas. *Post-hoc* analizei buvo naudojamos standartinės liekanos po to, kai statistinis reikšmingumas buvo patvirtintas chi kvadrato testu. Pasikliautiniesiems intervalams (PI) apskaičiuoti buvo naudojamas binominis metodas. Pearsono koreliacija buvo naudojama siekiant įvertinti ryšį tarp EEV paplitimo graužikų mėginiuose, kuriuose virusas aptiktas tik po pagausinimo ląstelių kultūroje, ir vidutinės oro temperatūros, kada buvo sugauti graužikai. Statistinė analizė ir vizualizacijos buvo atliktos naudojant programavimo kalbą R 4.3.2.

4.7. Finansavimas

Tyrimai buvo finansuoti Lietuvos sveikatos mokslų universiteto Mokslo fondo dotacijomis (Nr. V-789; Nr. V-786; Nr. 2021-V-0671; Nr. 2022-V-0352) ir Lietuvos mokslo tarybos lėšomis (Nr. S-MIP-23-80).

4.8. Etikos deklaracija

Smulkiųjų laukinių graužikų gaudymas buvo vykdomas gavus Aplinkos apsaugos agentūros leidimą ir Lietuvos Respublikos aplinkos ministerijos patvirtintas licencijas (Nr. 5 (2019-02-28) ir Nr. 12 (2020-02-27)). Visos procedūros ir graužikų nekropsija buvo atliekamos laikantis gyvūnų gerovės reikalavimų ir standartinių protokolų.

5. REZULTATAI

5.1. EEV paplitimas tarp šunų

Šiame darbe AT-PGR ir ELISA metodais buvo ištirti 473 šunų kraujo mėginiai. EEV RNR buvo nustatyta 18,6 proc. (88/473; 95 proc. PI 15,2–22,4) šunų kraujo / kraujo serumo mėginiuose ir 2 iš 3 CSS mėginiuose. Po ELISA tyrimo rezultatų patvirtinimo viruso neutralizacijos testu EEV specifiniai antikūnai buvo aptikti 21,6 proc. (102/473) (102/473; 95 proc. PI 17,9–25,6) šunų kraujo serumo mėginių.

Gaištamumo / eutanazijos dažnis šunų, kurių kraujyje aptikta EEV RNR, buvo 18,2 proc. (16/88; 95 proc. PI 10,8–27,8). Dėl sunkių neurologinių simptomų arba blogos ligos prognozės eutanazija buvo atlikta 12,5 proc. (11/88; 95 proc. PI 6,4–21,3) šunų, o likę 5,7 proc. (5/88; 95 proc. PI 1,9–12,8) nugaišo nepaisant intensyviosios priežiūros ir simptominio gydymo.

EEV specifiniai antikūnai buvo aptikti 26,1 proc. (23/88; 95 proc. PI 17,3–36,6) viremiškų šunų (vidutinis antikūnų kiekis – 189 NTU/ml; nuo 29,7 iki 525 NTU/ml). Šunų, kurių kraujyje tuo pačiu metu aptikta ir viruso RNR, ir jam specifinių antikūnų, amžiaus mediana buvo 8 metai (nuo 1 iki 14 metų). Šunų, vyresnių nei 1 metų, kraujyje EEV RNR ir jam specifinių antikūnų buvo aptikta statistiškai reikšmingai ($p < 0,01$) dažniau nei šunų iki 1 metų amžiaus (atitinkamai – 37,5 proc. (95 proc. PI 26,3–49,7) ir 0 proc.).

Beveik trečdaliui (29,6 proc. (26/88)) šunų, kurių kraujyje aptikta EEV RNR, šeimininkai pastebėjo įsisiurbusių erkių. Gretutinės erkių platinamų patogenų koinfekcijos buvo diagnozuotos 19,3 proc. (17/88; 95 proc. PI 11,7–29,1), iš jų 82,4 proc. (14/17) šunų nustatyta *Babesia* spp. koinfekcija, o likusiems 17,6 proc. (3/17)) – *A. phagocytophilum* koinfekcija.

Iš 88 šunų, kurių kraujyje nustatyta EEV RNR, buvo 48 patinai ir 40 patelių, o jų amžiaus mediana – 7 metai (nuo 2 mėnesių iki 15 metų). Statistinė

analizė atskleidė, jog statistiškai reikšmingai ($p < 0,01$) didesnę riziką neurologinių simptomų pasireiškimui turėjo patinai (43,7 proc. (95 proc. PI 29,5–58,8), palyginti su patelėmis (17,5 proc. (95 proc. PI 7,3–33,0). Taip pat buvo įvertinti šunų, kurių kraujyje aptikta EEV RNR, letalios baigties rizikos veiksniai. Nustatyta, jog vyresnio amžiaus šunys ($p < 0,01$) turėjo didesnę gaištamumo / eutanazijos riziką (vidutinis pasveikusių šunų amžius buvo 5,6 metų, o nugaišusių / eutanazuotų – 11,8 metų). Šunys, kuriems pasireiškė neurologinių simptomų (vidutinio sunkumo arba sunkių) turėjo statistiškai reikšmingai didesnę gaištamumo / eutanazijos riziką ($\chi^2 = 11,57$, df = 2, $p < 0,01$) (27,8 proc. (95 proc. PI 19,3–36,6) pasveikusių šunų, turėjusių neurologinių simptomų, ir 72,2 proc. (95 proc. PI 63,7–80,7)) pasveikusių šunų, neturėjusių neurologinių simptomų). Statistinė analizė atskleidė, jog šunys, kurių kraujo serume buvo aptikta EEV RNR ir EEV specifinių antikūnų (tuo pačiu metu pasireiškus ir neurologiniams simptomams), statistiškai reikšmingai ($\chi^2 = 5,05$, df = 1, $p < 0,05$) dažniau nugaišo / atlikta eutanazija nei šunys, kurių kraujyje aptikus EEV RNR, EEV specifinių antikūnų nebuvo rasta. Nustatyta, jog pasveikimo dažnis buvo 14,2 proc. mažesnis seroteigiamų ir 8,6 proc. didesnis seroneigiamų šunų grupėse, palyginti su pagal matematinį modelį tikėtinu pasveikimo dažniu. Priešingai, gaištamumo / eutanazijos dažnis buvo 65,5 proc. didesnis seroteigiamų ir 39,7 proc. maženis seroneigiamų šunų grupėse. Gauti rezultatai rodo, jog atidėtas arba nepakankamas imuninis atsakas bei EEV specifinių antikūnų gamyba, esant ūminei EE ligos eigai, kai neurologiniai simptomai pasireiškia esant viremijai, gali būti susiję su didesne šunų, sergančių EE gaištamumo / eutanazijos rizika.

Neurologiniai simptomai iš viso pasireiškė 31,8 proc. (28/88) šunų, kurių kraujyje aptikta EEV RNR. Sunkūs / vidutinio sunkumo neurologiniai simptomai pasireiškė 12,5 proc. (11/88) šunų – meningoencefalomielitui būdingi simptomai, tokie kaip tetraparezė, fascialinių refleksų stoka, regos sutrikimai, traukuliai, taip pat sąmonės sutrikimai ir elgsenos pokyčiai. Lengvi neurologiniai simptomai (pasunkėjęs atsistojimas ir judėjimas, atsisakymas judėti, sutrikusi, sukaustyta arba nekoordinuota eisena, kaklo srities hiperestezija) pasireiškė 19,3 proc. (17/88) šunų, kurių kraujyje aptikta EEV RNR.

Likusiems 68,2 proc. (60/88) šunų, kurių kraujyje nustatyta EEV RNR, buvo diagnozuota įvairių kitų ligų ir klinikinių požymių. Įvairios, nesusijusios ligos diagnozuotos 25 proc. (22/88), virškinimo trakto veiklos sutrikimai (vėmimas / viduriavimas) pasireiškė 37,5 proc. (33/88) (iš jų 17 proc. (15/88) taip pat turėjo neurologinių simptomų). Kitos erkių platinamos infekcijos diagnozuotos 13,6 proc. (12/88) (neįskaitant penkių šunų, turėjusių neurologinių simptomų). Nespecifiniai simptomai, tokie kaip apatija ir apetito praradimas, pasireiškė 11,3 proc. (10/88) šunų, kurių kraujyje aptikta EEV RNR.

5.2. EEV paplitimas tarp nuo šunų nurinktų erkių

Nuo šunų buvo nurinkta 117 erkių, kurių suspensijos buvo ištirtos AT-PGR metodu ir panaudotos „Neuro-2a“, „Vero“ ir „MARC-145“ ląstelių kultūrų užkrėtimui siekiant pagausinti viruso kiekį mėginiuose. EEV RNR buvo aptikta 34,2 proc. (40/117) erkių suspensijų mėginių. Viruso aptikimo dažnis statistiškai reikšmingai ($p = 0{,}01$) padidėjo po mėginio pagausinimo ląstelių kultūrose iki 56,4 proc. (66/117) „MARC-145“, 58,1 proc. (68/117) „Vero“ ir 60,6 proc. (71/117) „Neuro-2a“ ląstelių kultūrose po trijų serijinių persėjimų.

5.3. EEV paplitimas tarp smulkiųjų laukinių graužikų

Smulkiųjų laukinių graužikų smegenų ($n = 137$) ir vidaus organų ($n = 139$) mėginiai buvo ištirti AT-PGR tyrimo metodu. Gauti rezultatai atskleidė didelį EEV paplitimą viruso židiniuose sugautų graužikų mėginiuose. EEV RNR buvo nustatyta 104 (74,8 proc.; 95 proc. PI 66,7–81,1) graužikų suspensijose, iš jų 51,1 proc. (95 proc. PI 42,4–59,7) smegenų suspensijose ir 53,2 proc. (95 proc. PI 44,6–61,7) vidaus organų mišinio suspensijose. Aptikto NCR fragmento (126 bp dydžio) specifiškumas EEV buvo patvirtintas daline genomo sekoskaita (Supplementary material, Fig. S1).

EEV aptikimas statistiškai reikšmingai ($p < 0{,}01$) padidėjo po graužikų mėginių pagausinimo pelių neuroblastomos („Neuro-2a“) ląstelių kultūrose. EEV RNR buvo nustatyta 134 (96,4 proc. 95 proc. PI 91,8–98) graužikų izoliatuose ląstelių kultūrose, iš jų 85,4 proc. (95 proc. PI 78,4–90,9) smegenų izoliatuose ir 81,3 proc. (95 proc. PI 73,8–87,4) vidaus organų mišinio izoliatuose ląstelių kultūrose.

Visuose 19-oje tirtų EEV židinių buvo aptikta viruso RNR. EEV paplitimo dažnis svyravo nuo 25 iki 100 proc., remiantis tyrimais graužikų suspensijose. Po mėginio pagausinimo „Neuro-2a“ ląstelių kultūrose EEV RNR aptikimas statistiškai reikšmingai ($p < 0{,}01$) padidėjo iki 100 proc. 16-oje EEV židinių ir 71,4–90 proc. likusiuose 3-uose tirtuose židiniuose.

Nors nustatytas didelis EEV paplitimo dažnis tarp smulkiųjų laukinių graužikų, tačiau net 70,1 proc. smegenų ir vidaus organų suspensijų mėginių aptiktas mažas viruso kiekis, kurį indikuoja amplifikacija, fiksuojama tik 35–40 AT-PGR tyrimo cikle. Siekiant palyginti virusų kiekį prieš ir po mėginio kultivavimo „Neuro-2a“ ląstelių kultūroje, atrinktuose ($n = 30$) graužikų smegenų ir vidaus organų mišinio suspensijų bei jų izoliatų ląstelių kultūrų mėginiuose buvo išmatuotas virusinių dalelių kopijų kiekis. Vidutinis virusų kopijų kiekis statistiškai reikšmingai ($p < 0{,}05$) padidėjo po mėginio pagausinimo „Neuro-2a“ ląstelių kultūrose (nuo 5,71 $\log_{10}$ kopijų/ml iki 6,35 $\log_{10}$ kopijų/ml).

Graužikų smegenų ir vidaus organų mišinio mėginiai, kuriuose EEV RNR buvo aptikta tik po pagausinimo ląstelių kultūroje, buvo sugrupuoti pagal graužikų sugavimo mėnesį. Palyginus viruso aptikimo dažnumą ir vidutinę oro temperatūrą mėnesio, kada buvo sugauti graužikai, nustatyta stipri koreliacija ($r = 0{,}88$; $p < 0{,}05$) tarp viruso paplitimo dažnumo ir vidutinės oro temperatūros, kuri rodo, kad esant žemesnei oro temperatūrai EEV RNR aptikimo dažnis graužikų mėginiuose buvo didesnis.

Šiame darbe buvo ištirtas EEV paplitimas šešių skirtingų rūšių smulkiųjų graužikų, priklausančių *Muridae* ir *Cricetidae* šeimoms, mėginiuose. Statistinė analizė atskleidė, kad EEV RNR aptikimas buvo statistiškai reikšmingai ($p < 0{,}05$) dažnesnis paprastųjų pelėnų (*M. arvalis*) smegenų ir vidaus organų mišinio mėginių suspensijose, palyginti su kitomis rūšimis. Priešingai, EEV RNR aptikimas buvo statistiškai reikšmingai retesnis (Fišerio tikslusis kriterijus $p = 0{,}46$) geltonkaklių pelių (*A. flavicollis*) smegenų ir vidaus organų mišinio suspensijose. Po mėginių pagausinimo „Neuro-2a“ ląstelių kultūrose statistiškai reikšmingų EEV RNR aptikimo skirtumų tarp skirtingų graužikų rūšių nebuvo nustatyta.

IŠVADOS

1. EEV RNR nustatyta 18,6 proc. (95 proc. PI 15,2–22,4), o virusui specifinių antikūnų aptikta 21,6 proc. (95 proc. PI 17,9–25,6) tirtų šunų kraujo serumo mėginių.
2. Pagrindiniai šunų, kurių kraujyje aptikta EEV RNR, letalios baigties rizikos veiksniai buvo: vyresnis šuns amžius (vid. 11,8 m.; $p < 0{,}01$), neurologinių simptomų pasireiškimas ($p < 0{,}01$) ir EEV specifinių antikūnų buvimas serume ($p < 0{,}05$) esant viremijai ir pasireiškus sunkiems neurologiniams simptomams.
3. EEV RNR nustatymas nuo šunų nurinktose erkėse po viruso pagausinimo „MARC-145“, „Vero“ ir „Neuro-2a“ ląstelių kultūrose statistiškai reikšmingai ($p = 0{,}01$) padidėjo nuo 34,2 proc. (95 proc. PI 25,7–43,5) erkių suspensijose iki 56,4–60,7 proc. (95 proc. PI 46,9–69,6) ląstelių kultūrų izoliatuose.
4. EEV RNR nustatyta 51,1 proc. (95 proc. PI 42,4–59,7) graužikų smegenų ir 53,2 proc. (95 proc. PI 44,6–61,7) vidaus organų mišinio suspensijų mėginių. Virusas statistiškai reikšmingai dažniau buvo aptiktas *Microtus arvalis* ir rečiau *Apodemus flavicollis* rūšies graužikų mėginių suspensijose, palyginti su kitomis tirtomis graužikų rūšimis.

5. EEV RNR nustatymas graužikų mėginiuose po viruso pagausinimo „Neuro-2a“ ląstelių kultūrose statistiškai reikšmingai ($p < 0,01$) padidėjo nuo 74,8 proc. graužikų smegenų ir vidaus organų mišinio suspensijose iki 96,4 proc. (95 proc. PI 91,8–98) ląstelių kultūrų izoliatuose.
6. Nustatyta stipri koreliacija ($r = 0,88$; $p < 0,05$) tarp EEV RNR aptikimo dažnio graužikų mėginiuose, kuriuose virusas aptiktas tik po pagausinimo ląstelių kultūrose, ir vidutinės oro temperatūros, kai buvo sugauti graužikai, kuri rodo, kad esant žemesnei oro temperatūrai viruso aptikimo dažnis graužikų mėginiuose buvo statistiškai reikšmingai didesnis.

REFERENCES

1. Růžek D, Avšič Županc T, Borde J, Chrdle A, Eyer L, Karganova G, et al. Tick-borne encephalitis in Europe and Russia: Review of pathogenesis, clinical features, therapy, and vaccines. Antiviral Research. 2019 Apr;164:23–51.
2. Beauté J, Spiteri G, Warns-Petit E, Zeller H. Tick-borne encephalitis in Europe, 2012 to 2016. Euro Surveill. 2018 Nov;23(45):1800201.
3. Růžek D, Dobler G, Mantke OD. Tick-borne encephalitis: Pathogenesis and clinical implications. Travel Medicine and Infectious Disease. 2010 Jul;8(4):223–32.
4. Bogovič P. Tick-borne encephalitis: A review of epidemiology, clinical characteristics, and management. WJCC. 2015;3(5):430.
5. European Centre for Disease Prevention and Control. Tick-borne encephalitis. Annual epidemiological report for 2020. [Internet]. Stockhom: ECDC; 2022. [cited 2024 Feb 24]. Available from: https://www.ecdc.europa.eu/en/publications-data/tick-borne-encephalitis-annual-epidemiological-report-2020
6. Van Heuverswyn J, Hallmaier-Wacker LK, Beauté J, Gomes Dias J, Haussig JM, Busch K, et al. Spatiotemporal spread of tick-borne encephalitis in the EU/EEA, 2012 to 2020. [Internet]. Euro Surveill. 2023;28(11) [cited 2024 Jan 22]. Available from: https://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2023.28.11.2200543
7. Korenberg EI. Natural focality of infections: Current problems and prospects of research. Biol Bull Russ Acad Sci. 2010 Dec;37(7):665–76.
8. Borde JP, Kaier K, Hehn P, Matzarakis A, Frey S, Bestehorn M, et al. The complex interplay of climate, TBEV vector dynamics and TBEV infection rates in ticks –Monitoring a natural TBEV focus in Germany, 2009–2018. PLoS ONE. 2021 Jan;16(1): e0244668.
9. Randolph SE. Ticks and tick-borne disease systems in space and from space. Adv Parasitol. 2000;47:217-43.
10. Saegerman C, Humblet MF, Leandri M, Gonzalez G, Heyman P, Sprong H, et al. First Expert Elicitation of Knowledge on Possible Drivers of Observed Increasing Human Cases of Tick-Borne Encephalitis in Europe. Viruses. 2023 Mar;15(3):791.
11. Daniel M, Danielová V, Fialová A, Malý M, Kříž B, Nuttall PA. Increased Relative Risk of Tick-Borne Encephalitis in Warmer Weather. Front Cell Infect Microbiol. 2018 Mar;8:90.
12. Estrada-Peña A, Ayllón N, de la Fuente J. Impact of Climate Trends on Tick-Borne Pathogen Transmission. Front Physiol. 2012;3:64.
13. Semenza JC, Paz S. Climate change and infectious disease in Europe: Impact, projection and adaptation. The Lancet Regional Health – Europe. 2021 Oct;9:100230.
14. Voyiatzaki C, Papailia SI, Venetikou MS, Pouris J, Tsoumani ME, Papageorgiou EG. Climate Changes Exacerbate the Spread of *Ixodes ricinus* and the Occurrence of Lyme Borreliosis and Tick-Borne Encephalitis in Europe – How Climate Models Are Used as a Risk Assessment Approach for Tick-Borne Diseases. IJERPH. 2022 May;19(11): 6516.
15. Jaenson TG, Hjertqvist M, Bergström T, Lundkvist Å. Why is tick-borne encephalitis increasing? A review of the key factors causing the increasing incidence of human TBE in Sweden. Parasites Vectors. 2012 Aug;5(1):184.
16. Grochowska A, Milewski R, Pancewicz S, Dunaj J, Czupryna P, Milewska AJ, et al. Comparison of tick-borne pathogen prevalence in *Ixodes ricinus* ticks collected in urban areas of Europe. Sci Rep. 2020 Apr;10(1):6975.

17. Rizzoli A, Silaghi C, Obiegala A, Rudolf I, Hubálek Z, Földvári G, et al. *Ixodes ricinus* and Its Transmitted Pathogens in Urban and Peri-Urban Areas in Europe: New Hazards and Relevance for Public Health. Front Public Health. 2014 Dec;2:251.
18. Stefanoff P, Pfeffer M, Hellenbrand W, Rogalska J, Rühe F, Makówka A, et al. Virus Detection in Questing Ticks is not a Sensitive Indicator for Risk Assessment of Tick-Borne Encephalitis in Humans: TBEV Detection in Ticks to Assess TBE Risk. Zoonoses and Public Health. 2013 May;60(3):215–26.
19. Imhoff M, Hagedorn P, Schulze Y, Hellenbrand W, Pfeffer M, Niedrig M. Review: Sentinels of tick-borne encephalitis risk. Ticks and Tick-borne Diseases. 2015 Jul;6(5): 592–600.
20. Michelitsch A, Wernike K, Klaus C, Dobler G, Beer M. Exploring the Reservoir Hosts of Tick-Borne Encephalitis Virus. Viruses. 2019 Jul;11(7):669.
21. Mikryukova TP, Moskvitina NS, Kononova YV, Korobitsyn IG, Kartashov MY, Tyuten Kov OY, et al. Surveillance of tick-borne encephalitis virus in wild birds and ticks in Tomsk city and its suburbs (Western Siberia). Ticks and Tick-borne Diseases. 2014 Mar;5(2):145–51.
22. Knap N, Korva M, Dolinšek V, Sekirnik M, Trilar T, Avšič-Županc T. Patterns of Tick-Borne Encephalitis Virus Infection in Rodents in Slovenia. Vector-Borne and Zoonotic Diseases. 2012 Mar;12(3):236–42.
23. Pérez D, Kneubühler Y, Rais O, Gern L. Seasonality of *Ixodes ricinus* Ticks on Vegetation and on Rodents and *Borrelia burgdorferi* sensu lato Genospecies Diversity in Two Lyme Borreliosis–Endemic Areas in Switzerland. Vector-Borne and Zoonotic Diseases. 2012 Aug;12(8):633–44.
24. Krawczuk K, Czupryna P, Pancewicz S, Ołdak E, Moniuszko-Malinowska A. Comparison of tick-borne encephalitis between children and adults – analysis of 669 patients. J Neurovirol. 2020 Aug;26(4):565–71.
25. Bakhvalova VN, Dobrotvorsky AK, Panov VV, Matveeva VA, Tkachev SE, Morozova OV. Natural Tick-Borne Encephalitis Virus Infection among Wild Small Mammals in the Southeastern Part of Western Siberia, Russia. Vector-Borne and Zoonotic Diseases. 2006 Mar;6(1):32–41.
26. Tonteri E, Jääskeläinen AE, Tikkakoski T, Voutilainen L, Niemimaa J, Henttonen H, et al. Tick-borne Encephalitis Virus in Wild Rodents in Winter, Finland, 2008–2009. Emerg Infect Dis. 2011 Jan;17(1):72–5.
27. Süss J. Epidemiology and ecology of TBE relevant to the production of effective vaccines. Vaccine. 2003 Apr;21:S19–35.
28. Salat J, Růžek D. Tick-borne encephalitis in domestic animals. Acta Virol. 2020; 64(02):226–32.
29. Pfeffer M, Dobler G. Tick-borne encephalitis virus in dogs – is this an issue? Parasites & Vectors. 2011 Apr;4(1):59.
30. Kleeb C, Golini L, Beckmann K, Torgerson P, Steffen F. Canine Tick-Borne Encephalitis: Clinical Features, Survival Rate and Neurological Sequelae: A Retrospective Study of 54 Cases (1999–2016). Front Vet Sci. 2021 Nov;8:782044.
31. Leschnik MW, Kirtz GC, Thalhammer JG. Tick-borne encephalitis (TBE) in dogs. International Journal of Medical Microbiology. 2002 Jun;291:66–9.
32. Dultz R, Goldhammer M. Tick-borne encephalitis in a dog. Tierarztl Prax Ausg K Kleintiere Heimtiere. 2021 Oct;49(05):377–81.
33. Andersson E, Kendall A, Url A, Auer A, Leschnik M. The first RT-qPCR confirmed case of tick-borne encephalitis in a dog in Scandinavia. Acta Vet Scand. 2020 Dec;62(1):51.

34. Bajer A, Rodo A, Bednarska M, Mierzejewska E, Welc-Falęciak R. *Babesia canis* and tick-borne encephalitis virus (TBEV) co-infection in a sled dog. Ann Agric Environ Med. 2013;20(3):426–30.
35. Holzmann H. Diagnosis of tick-borne encephalitis. Vaccine. 2003 Apr;21:S36–40.
36. European Centre for Disease Prevention and Control Factsheet about tick-borne encephalitis (TBE). [Internet]. 2024 ECDC. [cited 2024 Mar 7] Available from: https://www.ecdc.europa.eu/en/tick-borne-encephalitis/facts/factsheet
37. Vilibic-Cavlek T, Ferenc T, Vujica Ferenc M, Bogdanic M, Potocnik-Hunjadi T, Sabadi D, et al. Cross-Reactive Antibodies in Tick-Borne Encephalitis: Case Report and Literature Review. Antibodies. 2022 Nov;11(4):72.
38. Chiffi G, Grandgirard D, Leib SL, Chrdle A, Růžek D. Tick-borne encephalitis: A comprehensive review of the epidemiology, virology, and clinical picture. Reviews in Medical Virology. 2023 Sep;33(5):e2470.
39. The ICTV Report on Virus Classification and Taxon Nomenclature. [Internet]. [cited 2024 Mar 7] Available from: https://ictv.global/report/chapter/flaviviridae/flaviviridae/orthoflavivirus
40. Pulkkinen LIA, Butcher SJ, Anastasina M. Tick-Borne Encephalitis Virus: A Structural View. Viruses. 2018;28;10(7).
41. Lindenbach BD, Murray CL, Thiel HJ & Rice CM. In: Knippe DM. editor. Flaviviridae. Fields Virology, 6th ed.; Lippincott Williams & Wilkins, Philadelphia, PA, USA; 2013. p. 712-746.
42. Füzik T, Formanová P, Růžek D, Yoshii K, Niedrig M, Plevka P. Structure of tick-borne encephalitis virus and its neutralization by a monoclonal antibody. Nat Commun. 2018 Jan;9(1):1–11.
43. Tkachev SE, Chicherina GS, Golovljova I, Belokopytova PS, Tikunov AY, Zadora OV, et al. New genetic lineage within the Siberian subtype of tick-borne encephalitis virus found in Western Siberia, Russia. Infect Gen Evol. 2017 Dec;56:36–43.
44. Lorenz IC, Kartenbeck J, Mezzacasa A, Allison SL, Heinz FX, Helenius A. Intracellular Assembly and Secretion of Recombinant Subviral Particles from Tick-Borne Encephalitis Virus. J Virol. 2003 Apr;77(7):4370–82.
45. Heinz FX, Allison SL. Flavivirus Structure and Membrane Fusion. Adv Virus Res. 2003;59:63-97.
46. Ecker M, Allison SL, Meixner T, Heinz FX. Sequence analysis and genetic classification of tick-borne encephalitis viruses from Europe and Asia. Journal of General Virology. 1999 Jan;80(1):179–85.
47. Zlobin VI, Demina TV, Mamaev LV, Butina TV, Belikov SI, Gorin OZ, et al. Analysis of genetic variability of strains of tick-borne encephalitis virus by primary structure of a fragment of the membrane protein E gene. Vopr Virusol. 2001;46(1):12–6.
48. Demina TV, Dzhioev IP, Kozlova IV, Verkhozina MM, Tkachev SE, Doroshchenko EK, et al. Genotypes 4 and 5 of the tick-borne encephalitis virus: features of the genome structure and possible scenario for its formation. Vopr Virusol. 2012;57(4):13–9.
49. Dai X, Shang G, Lu S, Yang J, Xu J. A new subtype of eastern tick-borne encephalitis virus discovered in Qinghai-Tibet Plateau, China. Emerging Microbes & Infections. 2018 Dec;7(1):1–9.
50. Adelshin RV, Sidorova EA, Bondaryuk AN, Trukhina AG, Sherbakov DYu, White Iii RA, et al. “886-84-like” tick-borne encephalitis virus strains: Intraspecific status elucidated by comparative genomics. Ticks and Tick-borne Diseases. 2019 Aug;10(5):1168–72.

51. Bondaryuk AN, Sidorova EA, Adelshin RV, Andaev EI, Balakhonov SV. Reporting of new tick-borne encephalitis virus strains isolated in Eastern Siberia (Russia) in 1960–2011 and explaining them in an evolutionary context using Bayesian phylogenetic inference. Ticks and Tick-borne Diseases. 2020 Sep;11(5):101496.
52. Kovalev SY, Mukhacheva TA. Reconsidering the classification of tick-borne encephalitis virus within the Siberian subtype gives new insights into its evolutionary history. Infect Gen Evol. 2017 Nov;55:159–65.
53. Kozlova IV, Demina TV, Tkachev SE, Doroshchenko EK, Lisak OV, Verkhozina MM, et al. Characteristics of the Baikal subtype of Tick-borne encephalitis virus circulating in Eastern Siberia. Acta Biomedica Scientifica. 2018 Jul;3(4):53–60.
54. Demina TV, Tkachev SE, Kozlova IV, Doroshchenko EK, Lisak OV, Suntsova OV, et al. Comparative analysis of complete genome sequences of European subtype tick-borne encephalitis virus strains isolated from *Ixodes persulcatus* ticks, long-tailed ground squirrel (*Spermophilus undulatus*), and human blood in the Asian part of Russia. Ticks and Tick-borne Diseases. 2017 Jun;8(4):547–53.
55. Rieille N, Bressanelli S, Freire CCM, Arcioni S, Gern L, Péter O, et al. Prevalence and phylogenetic analysis of tick-borne encephalitis virus (TBEV) in field-collected ticks (*Ixodes ricinus*) in southern Switzerland. Parasit Vectors. 2014;7(1):443.
56. Lommano E, Burri C, Maeder G, Guerne M, Bastic V, Patalas E, et al. Prevalence and Genotyping of Tick-Borne Encephalitis Virus in Questing *Ixodes ricinus* Ticks in a New Endemic Area in Western Switzerland. J Med Entomol. 2012 Jan;49(1):156–64.
57. Uzcátegui NY, Sironen T, Golovljova I, Jääskeläinen AE, Välimaa H, Lundkvist Å, et al. Rate of evolution and molecular epidemiology of tick-borne encephalitis virus in Europe, including two isolations from the same focus 44 years apart. Journal of General Virology. 2012 Apr;93(4):786–96.
58. Dekker M, Laverman GD, de Vries A, Reimerink J, Geeraedts F. Emergence of tick-borne encephalitis (TBE) in the Netherlands. Ticks and Tick-borne Diseases. 2019 Jan;10(1):176–9.
59. Hayasaka D, Suzuki Y, Kariwa H, Ivanov L, Volkov V, Demenev V, et al. Phylogenetic and virulence analysis of tick-borne encephalitis viruses from Japan and far-eastern Russia. Journal of General Virology. 1999 Dec;80(12):3127–35.
60. Zlobin VI, Demina TV, Belikov SI, Butina TV, Gorin OZ, Adel'shin RV, et al. Genetic typing of tick-borne encephalitis virus based on an analysis of the levels of homology of a membrane protein gene fragment. Vopr Virusol. 2001;46(1):17–22.
61. Subbotina EL, Loktev VB. Molecular evolution of the tick-borne encephalitis and Powassan viruses. Mol Biol. 2012 Feb;46(1):75–84.
62. Deviatkin AA, Karganova GG, Vakulenko YA, Lukashev AN. TBEV Subtyping in Terms of Genetic Distance. Viruses. 2020 Oct;12(11):1240.
63. Charrel RN, Attoui H, Butenko AM, Clegg JC, Deubel V, Frolova TV, et al. Tick-borne virus diseases of human interest in Europe. Clinical Microbiology and Infection. 2004 Dec;10(12):1040–55.
64. Deviatkin AA, Kholodilov IS, Vakulenko YA, Karganova GG, Lukashev AN. Tick-Borne Encephalitis Virus: An Emerging Ancient Zoonosis? Viruses. 2020 Feb;12(2):247.
65. Smura T, Tonteri E, Jääskeläinen A, Von Troil G, Kuivanen S, Huitu O, et al. Recent establishment of tick-borne encephalitis foci with distinct viral lineages in the Helsinki area, Finland. Emerging Microbes & Infections. 2019 Jan;8(1):675–83.
66. Yoshii K, Song JY, Park SB, Yang J, Schmitt HJ. Tick-borne encephalitis in Japan, Republic of Korea and China: TBE in Japan, ROK and China. Emerging Microbes & Infections. 2017 Jan;6(1):1–10.

67. Yurchenko OO, Dubina DO, Vynograd NO, Gonzalez JP. Partial Characterization of Tick-Borne Encephalitis Virus Isolates from Ticks of Southern Ukraine. Vector-Borne and Zoonotic Diseases. 2017 Aug;17(8):550–7.
68. Lundkvist Å, Vene S, Golovljova I, Mavtchoutko V, Forsgren M, Kalnina V, et al. Characterization of tick-borne encephalitis virus from Latvia: evidence for co-circulation of three distinct subtypes. Journal of Medical Virology. 2001 Dec;65(4):730–5.
69. Golovljova I, Vene S, Sjölander KB, Vasilenko V, Plyusnin A, Lundkvist Å. Characterization of tick-borne encephalitis virus from Estonia. Journal of Medical Virology. 2004 Dec;74(4):580–8.
70. Kim SY, Yun SM, Han MG, Lee IY, Lee NY, Jeong YE, et al. Isolation of Tick-Borne Encephalitis Viruses from Wild Rodents, South Korea. Vector-Borne and Zoonotic Diseases. 2008 Feb;8(1):7–14.
71. Muto M, Bazartseren B, Tsevel B, Dashzevge E, Yoshii K, Kariwa H. Isolation and characterization of tick-borne encephalitis virus from *Ixodes persulcatus* in Mongolia in 2012. Ticks and Tick-borne Diseases. 2015 Jul;6(5):623–9.
72. Briggs BJ, Atkinson B, Czechowski DM, Larsen PA, Meeks HN, Carrera JP, et al. Tick-Borne Encephalitis Virus, Kyrgyzstan. Emerg Infect Dis. 2011 May;17(5):876–9.
73. Pukhovskaya NM, Morozova OV, Belozerova NB, Bakhmetyeva SV, Vysochina NP, Zdanovskaya NI, et al. Comparative analysis of genomes of tick-borne encephalitis virus strains isolated from mosquitoes and ticks. Vopr Virusol. 2017;62(1):30–5.
74. Jaaskelainen AE, Sironen T, Murueva GB, Subbotina N, Alekseev AN, Castren J, et al. Tick-borne encephalitis virus in ticks in Finland, Russian Karelia and Buryatia. Journal of General Virology. 2010 Nov;91(11):2706–12.
75. Tkachev SE, Babkin IV, Chicherina GS, Kozlova IV, Verkhozina MM, Demina TV, et al. Genetic diversity and geographical distribution of the Siberian subtype of the tick-borne encephalitis virus. Ticks and Tick-borne Diseases. 2020 Mar;11(2):101327.
76. Ponomareva EP, Mikryukova TP, Gori AV, Kartashov MY, Protopopova EV, Chausov EV, et al. Detection of Far-Eastern subtype of tick-borne encephalitis viral RNA in ticks collected in the Republic of Moldova. J Vector Borne Dis. 2015 Dec;52(4):334–6.
77. Brabec M, Daniel M, Malý M, Danielová V, Kříž B, Kott I, et al. Analysis of meteorological effects on the incidence of tick-borne encephalitis in the Czech Republic over a thirty-year period. Virol Res Rev 2017 Mar;1(1).
78. Martello E, Mannelli A, Ragagli C, Ambrogi C, Selmi M, Ceballos LA, et al. Range expansion of *Ixodes ricinus* to higher altitude, and co-infestation of small rodents with *Dermacentor marginatus* in the Northern Apennines, Italy. Ticks and Tick-borne Diseases. 2014 Oct;5(6):970–4.
79. Soleng A, Edgar KS, Paulsen KM, Pedersen BN, Okbaldet YB, Skjetne IEB, et al. Distribution of *Ixodes ricinus* ticks and prevalence of tick-borne encephalitis virus among questing ticks in the Arctic Circle region of northern Norway. Ticks and Tick-borne Diseases. 2018 Jan;9(1):97–103.
80. Esser HJ, Lim SM, de Vries A, Sprong H, Dekker DJ, Pascoe EL, et al. Continued Circulation of Tick-Borne Encephalitis Virus Variants and Detection of Novel Transmission Foci, the Netherlands. Emerg Infect Dis. 2022 Dec;28(12):2416–24.
81. De Graaf JA, Reimerink JHJ, Voorn GP, Bij de Vaate EA, de Vries A, Rockx B, et al. First human case of tick-borne encephalitis virus infection acquired in the Netherlands, July 2016. Euro Surveill. 2016 Aug;21(33):30318.
82. Holding M, Dowall SD, Medlock JM, Carter DP, Pullan ST, Lewis J, et al. Tick-Borne Encephalitis Virus, United Kingdom. Emerg Infect Dis. 2020 Jan;26(1):90–6.

83. Velay A, Solis M, Kack-Kack W, Gantner P, Maquart M, Martinot M, et al. A new hot spot for tick-borne encephalitis (TBE): A marked increase of TBE cases in France in 2016. Ticks and Tick-borne Diseases. 2018 Jan;9(1):120–5.
84. Agergaard CN, Rosenstierne MW, Bødker R, Rasmussen M, Andersen PHS, Fomsgaard A. New tick-borne encephalitis virus hot spot in Northern Zealand, Denmark, October 2019. Euro Surveill. 2019;24(43):1900639.
85. Topp AK, Springer A, Dobler G, Bestehorn-Willmann M, Monazahian M, Strube C. New and Confirmed Foci of Tick-Borne Encephalitis Virus (TBEV) in Northern Germany Determined by TBEV Detection in Ticks. Pathogens. 2022 Jan;11(2):126.
86. Lang D, Chitimia-Dobler L, Bestehorn-Willmann M, Lindau A, Drehmann M, Stroppel G, et al. The Emergence and Dynamics of Tick-Borne Encephalitis Virus in a New Endemic Region in Southern Germany. Microorganisms. 2022 Oct;10(11):2125.
87. Bauer BU, Könenkamp L, Stöter M, Wolf A, Ganter M, Steffen I, et al. Increasing awareness for tick-borne encephalitis virus using small ruminants as suitable sentinels: Preliminary observations. One Health. 2021 Jun;12:100227.
88. Wallenhammar A, Lindqvist R, Asghar N, Gunaltay S, Fredlund H, Davidsson Å, et al. Revealing new tick-borne encephalitis virus foci by screening antibodies in sheep milk. Parasites Vectors. 2020 Dec;13(1):185.
89. Süss J. Tick-borne encephalitis 2010: Epidemiology, risk areas, and virus strains in Europe and Asia – An overview. Ticks and Tick-borne Diseases. 2011 Mar;2(1):2–15.
90. Chitimia-Dobler L, Rieß R, Kahl O, Wölfel S, Dobler G, Nava S, et al. *Ixodes inopinatus* − Occurring also outside the Mediterranean region. Ticks and Tick-borne Diseases. 2018 Feb;9(2):196–200.
91. Estrada-Peña A, Nava S, Petney T. Description of all the stages of *Ixodes inopinatus* n. sp. (*Acari: Ixodidae*). Ticks and Tick-borne Diseases. 2014 Oct;5(6):734–43.
92. Korenberg EI, Sirotkin MB, Kovalevskii YV. Adaptive Features of the Biology of Closely Related Species of *Ixodid* Ticks That Determine Their Distribution (Illustrated on the Example of the Taiga Tick *Ixodes persulcatus* Sch. 1930 and the Castor Bean Tick *Ixodes ricinus* L. 1758). Biol Bull Rev. 2021 Nov;11(6):602–15.
93. Kholodilov I, Belova O, Burenkova L, Korotkov Y, Romanova L, Morozova L, et al. Ixodid ticks and tick-borne encephalitis virus prevalence in the South Asian part of Russia (Republic of Tuva). Ticks and Tick-borne Diseases. 2019 Aug;10(5):959–69.
94. Rubel F, Brugger K, Walter M, Vogelgesang JR, Didyk YM, Fu S, et al. Geographical distribution, climate adaptation and vector competence of the Eurasian hard tick *Haemaphysalis concinna*. Ticks and Tick-borne Diseases. 2018 Jul;9(5):1080–9.
95. Biernat B, Karbowiak G, Werszko J, Stańczak J. Prevalence of tick-borne encephalitis virus (TBEV) RNA in *Dermacentor reticulatus* ticks from natural and urban environment, Poland. Exp Appl Acarol. 2014 Dec;64(4):543–51.
96. Mierzejewska EJ, Pawełczyk A, Radkowski M, Welc-Faleciak R, Bajer A. Pathogens vectored by the tick, *Dermacentor reticulatus*, in endemic regions and zones of expansion in Poland. Parasites Vectors. 2015 Dec;8(1):490.
97. Zając V, Wójcik-Fatla A, Sawczyn A, Cisak E, Sroka J, Kloc A, et al. Prevalence of infections and co-infections with 6 pathogens in *Dermacentor reticulatus* ticks collected in eastern Poland. Ann Agric Environ Med. 2017 Mar;24(1):26–32.
98. Chitimia-Dobler L, Lemhöfer G, Król N, Bestehorn M, Dobler G, Pfeffer M. Repeated isolation of tick-borne encephalitis virus from adult *Dermacentor reticulatus* ticks in an endemic area in Germany. Parasites Vectors. 2019 Dec;12(1):90.

99. Ličková M, Fumačová Havlíková S, Sláviková M, Slovák M, Drexler JF, Klempa B. *Dermacentor reticulatus* is a vector of tick-borne encephalitis virus. Ticks and Tick-borne Diseases. 2020 Mar;101414.
100. Belova OA, Litov AG, Kholodilov IS, Kozlovskaya LI, Bell-Sakyi L, Romanova LI, et al. Properties of the tick-borne encephalitis virus population during persistent infection of ixodid ticks and tick cell lines. Ticks and Tick-borne Diseases. 2017 Oct;8(6): 895–906.
101. Labuda M, Randolph SE. Survival strategy of tick-borne encephalitis virus: Cellular basis and environmental determinants. Zentralblatt für Bakteriologie. 1999 Dec;289(5–7):513–24.
102. Kahl O, Gray JS. The biology of *Ixodes ricinus* with emphasis on its ecology. Ticks and Tick-borne Diseases. 2023 Mar;14(2):102114.
103. Randolph SE. Tick ecology: processes and patterns behind the epidemiological risk posed by ixodid ticks as vectors. Parasitology. 2004 Oct;129(S1):S37–65.
104. Randolph SE, Green RM, Hoodless AN, Peacey MF. An empirical quantitative framework for the seasonal population dynamics of the tick *Ixodes ricinus*. International Journal for Parasitology. 2002 Jul;32(8):979–89.
105. Gray JS. Mating and behavioural diapause in *Ixodes ricinus* L. Exp Appl Acarol. 1987 Mar;3(1):61–71.
106. Gray JS, Kahl O, Lane RS, Levin ML, Tsao JI. Diapause in ticks of the medically important *Ixodes ricinus* species complex. Ticks and Tick-borne Diseases. 2016 Jul;7(5):992–1003.
107. Cayol C, Koskela E, Mappes T, Siukkola A, Kallio ER. Temporal dynamics of the tick *Ixodes ricinus* in northern Europe: epidemiological implications. Parasites Vectors. 2017 Dec;10(1):166.
108. Perret JL, Guigoz E, Rais O, Gern L. Influence of saturation deficit and temperature on *Ixodes ricinus* tick questing activity in a Lyme borreliosis-endemic area (Switzerland). Parasitology Research. 2000 Jun;86(7):554–7.
109. Randolph SE. The shifting landscape of tick-borne zoonoses: tick-borne encephalitis and Lyme borreliosis in Europe. Philos Trans R Soc Lond B Biol Sci. 2001 Jul; 356(1411):1045-56.
110. Lees AD. The Sensory Physiology of the Sheep Tick, *Ixodes ricinus* L. Journal of Experimental Biology. 1948 Jun;25(2):145–207.
111. Hvidsten D, Frafjord K, Gray JS, Henningsson AJ, Jenkins A, Kristiansen BE, et al. The distribution limit of the common tick, *Ixodes ricinus*, and some associated pathogens in north-western Europe. Ticks and Tick-borne Diseases. 2020 Jul;11(4):101388.
112. Lees AD. The water balance in *Ixodes ricinus* L. and certain other species of ticks. Parasitology. 1946 Jan;37(1–2):1–20.
113. Kahl O, Knülle W. Water vapour uptake from subsaturated atmospheres by engorged immature ixodid ticks. Exp Appl Acarol. 1988 Feb;4(1):73–83.
114. Perret JL, Rais O, Gern L. Influence of Climate on the Proportion of *Ixodes ricinus* Nymphs and Adults Questing in a Tick Population. J Med Entomol. 2004 May ;41(3):361–5.
115. Ehrmann S, Liira J, Gärtner S, Hansen K, Brunet J, Cousins SAO, et al. Environmental drivers of *Ixodes ricinus* abundance in forest fragments of rural European landscapes. BMC Ecol. 2017 Dec;17(1):31.
116. Hansford KM, Wheeler BW, Tschirren B, Medlock JM. Questing *Ixodes ricinus* ticks and *Borrelia* spp. in urban green space across Europe: A review. Zoonoses and Public Health. 2022 May;69(3):153–66.

117. Dantas-Torres F, Otranto D. Seasonal dynamics of *Ixodes ricinus* on ground level and higher vegetation in a preserved wooded area in southern Europe. Veterinary Parasitology. 2013 Feb;192(1–3):253–8.
118. Gandy SL, Hansford KM, Medlock JM. Possible expansion of *Ixodes ricinus* in the United Kingdom identified through the Tick Surveillance Scheme between 2013 and 2020. Medical Vet Entomology. 2023 Mar;37(1):96–104.
119. Estrada-Peña A. Ticks as vectors: taxonomy, biology and ecology. Rev Sci Tech OIE. 2015 Apr;34(1):53–65.
120. Walker AR, Alberdi MP, Urquhart KA, Rose H. Risk factors in habitats of the tick *Ixodes ricinus* influencing human exposure to *Ehrlichia phagocytophila* bacteria. Medical Vet Entomology. 2001 Mar;15(1):40–9.
121. Jouda F, Perret JL, Gern L. *Ixodes ricinus* Density, and Distribution and Prevalence of *Borrelia burgdorferi* Sensu Lato Infection Along an Altitudinal Gradient. J Med Entomol. 2004 Mar;41(2):162–9.
122. Ferquel E, Garnier M, Marie J, Bernède-Bauduin C, Baranton G, Pérez-Eid C, et al. Prevalence of *Borrelia burgdorferi* Sensu Lato and *Anaplasmataceae* Members in *Ixodes ricinus* Ticks in Alsace, a Focus of Lyme Borreliosis Endemicity in France. Appl Environ Microbiol. 2006 Apr;72(4):3074–8.
123. Boyard C, Barnouin J, Gasqui P, Vourc'h G. Local environmental factors characterizing *Ixodes ricinus* nymph abundance in grazed permanent pastures for cattle. Parasitology. 2007 Jul;134(7):987–94.
124. Gray JS, Kahl O, Janetzki C, Stein J, Guy E. The spatial distribution of *Borrelia burgdorferi*-infected *Ixodes ricinus* in the Connemara region of County Galway, Ireland. Exp Appl Acarol. 1995 Mar;19(3):163–72.
125. Gilot B, Pautou G, Moncada E. [Vegetation analysis used for the detection of exophile tick populations in the south-east of France: the example *Ixodes ricinus* (Linne 1758) (*acarina, ixodoidea*)]. Acta Trop. 1975;32(4):340–7.
126. Goldstein V, Boulanger N, Schwartz D, George JC, Ertlen D, Zilliox L, et al. Factors responsible for *Ixodes ricinus* nymph abundance: Are soil features indicators of tick abundance in a French region where Lyme borreliosis is endemic? Ticks and Tick-borne Diseases. 2018 May;9(4):938–44.
127. Balestrieri A, Remonti L, Morotti L, Saino N, Prigioni C, Guidali F. Multilevel habitat preferences of *Apodemus sylvaticus* and *Clethrionomys glareolus* in an intensively cultivated agricultural landscape. Ethology Ecology & Evolution. 2017 Jan;29(1):38–53.
128. Krawczyk AI, van Duijvendijk GLA, Swart A, Heylen D, Jaarsma RI, Jacobs FHH, et al. Effect of rodent density on tick and tick-borne pathogen populations: consequences for infectious disease risk. Parasites Vectors. 2020 Dec;13(1):34.
129. Tkadlec E, Václavík T, Široký P. Rodent Host Abundance and Climate Variability as Predictors of Tickborne Disease Risk 1 Year in Advance. Emerg Infect Dis. 2019 Sep; 25(9):1738–41.
130. Labuda M, Kozuch O, Zuffová E, Elecková E, Hails RS, Nuttall PA. Tick-Borne Encephalitis Virus Transmission between Ticks Cofeeding on Specific Immune Natural Rodent Hosts. Virology. 1997 Aug;235(1):138–43.
131. Tattersall FH, Macdonald DW, Hart BJ, Manley WJ, Feber RE. Habitat use by wood mice (*Apodemus sylvaticus*) in a changeable arable landscape. Journal of Zoology. 2001 Dec;255(4):487–94.
132. Gerth HJ, Grimshandl D, Stage B, Döller G, Kunz C. Roe deer as sentinels for endemicity of tick-borne encephalitis virus. Epidemiol Infect. 1995 Oct;115(2):355–65.

133. Friberg Holm M. Tick-borne diseases in roe deer – seroprevalence, possible cut-off and effects on health. 2023. Second cycle, A2E. Uppsala: SLU, Dept. of Biomedical Sciences and Veterinary Public Health.
134. Crooks E, Randolph SE. Walking by *Ixodes ricinus* ticks: intrinsic and extrinsic factors determine the attraction of moisture or host odour. J Exp Biol. 2006 Jun;209(11):2138–42.
135. Donzé G, McMahon C, Guerin PM. Rumen metabolites serve ticks to exploit large mammals. J Exp Biol. 2004 Nov;207(24):4283–9.
136. Van Duijvendijk G, Gort G, Sprong H, Takken W. Behavioural responses of *Ixodes ricinus* nymphs to carbon dioxide and rodent odour. Medical Vet Entomology. 2017 Jun;31(2):220–3.
137. Carroll JF. How Specific are Host-Produced Kairomones to Host-Seeking Ixodid Ticks? Exp Appl Acarol. 2002;28(1–4):155–61.
138. World Health Organization Technical Report Series No. 369. Arboviruses and Human Disease. Geneva, 1967 [Internet]. [cited 2024 Mar 8] Available from: https://iris.who.int/bitstream/handle/10665/40664/WHO_TRS_369.pdf;jsessionid=BF8677BAD3BBA7BA298BB96CB669FD35?sequence=1
139. Turell MJ. Horizontal and vertical transmission of viruses by insect and tick vectors. The Arboviruses: Epidemiology and Ecology, London: CRC Press, 2020. p. 127–152.
140. Nuttall PA, Labuda M. Dynamics of infection in tick vectors and at the tick–host interface. Adv Virus Res. 2003;60:233-72.
141. Kozuch O, Gresíková M, Nosek J, Lichard M, Sekeyová M. The role of small rodents and hedgehogs in a natural focus of tick-borne encephalitis. Bull World Health Organ. 1967;36 Suppl:61–6.
142. Karabatsos N. International Catalogue of Arboviruses. Including certain other viruses of vertebrates. 3rd ed. San Antonio, Tex: Published for the Subcommittee on Information Exchange of the American Committee on Arthropod-borne Viruses by the American Society of Tropical Medicine and Hygiene, 1985.
143. Randolph SE, Gern L, Nuttall PA. Co-feeding ticks: Epidemiological significance for tick-borne pathogen transmission. Parasitology Today. 1996 Dec;12(12):472–9.
144. Morozova OV, Panov VV, Bakhvalova VN. Innate and adaptive immunity in wild rodents spontaneously and experimentally infected with the tick-borne encephalitis virus. Infect Genet Evol. 2020 Jun;80:104187.
145. Michelitsch A, Tews BA, Klaus C, Bestehorn-Willmann M, Dobler G, Beer M, et al. *In vivo* characterization of tick-borne encephalitis virus in bank voles (*Myodes glareolus*). Viruses. 2019 Nov;11(11):1069.
146. Randolph SE. Transmission of tick-borne pathogens between co-feeding ticks: Milan Labuda's enduring paradigm. Ticks and Tick-borne Diseases. 2011 Dec;2(4):179–82.
147. Hartemink NA, Randolph SE, Davis SA, Heesterbeek JAP. The Basic Reproduction Number for Complex Disease Systems: Defining R_0 for Tick-Borne Infections. The American Naturalist. 2008 Jun;171(6):743–54.
148. Šimo L, Kazimirova M, Richardson J, Bonnet SI. The Essential Role of Tick Salivary Glands and Saliva in Tick Feeding and Pathogen Transmission. Front Cell Infect Microbiol. 2017 Jun;7:281.
149. Nuttall PA, Labuda M. Tick–host interactions: saliva-activated transmission. Parasitology. 2004 Oct;129(S1):S177–89.
150. Danielová V, Holubová J, Pejčoch M, Daniel M. Potential significance of transovarial transmission in the circulation of tick-borne encephalitis virus. Folia Parasit. 2002 Dec;49(4):323–5.

151. Chitimia-Dobler L, Mackenstedt U, Kahl O. Chapter 3: Transmission/Natural cycle. Tick-borne encephalitis – The Book. 2019. [Internet]. [cited 2024 May 1]; Available from: https://tbenews.com/tbe/tbe3/
152. Labuda M, Nuttall PA. Tick-borne viruses. Parasitology. 2004 Oct;129(S1):S221–45.
153. Nonaka E, Ebel GD, Wearing HJ. Persistence of Pathogens with Short Infectious Periods in Seasonal Tick Populations: The Relative Importance of Three Transmission Routes. PLoS ONE. 2010 Jul;5(7):e11745.
154. Nuttall PA. Tick-borne viruses. In: Sonenshine DE, Roe RM, editors. Biology of ticks, Vol. 2. NY: Oxford University Press, 2014. p. 180-210.
155. Alekseev AN. Ecology of tick-borne encephalitis virus: part of *Ixodidae* ticks males in its circulation. Ecol Parasitol. 1992;1:48-58.
156. Pettersson JHO, Golovljova I, Vene S, Jaenson TG. Prevalence of tick-borne encephalitis virus in *Ixodes ricinus* ticks in northern Europe with particular reference to Southern Sweden. Parasites Vectors. 2014 Dec;7(1):102.
157. Krebs CJ. Population Fluctuations in Rodents. University of Chicago Press; 2013.
158. Mihalca AD, Sándor AD. The role of rodents in the ecology of *Ixodes ricinus* and associated pathogens in Central and Eastern Europe. Front Cell Infect Microbiol. 2013 Oct;3:56.
159. Richter D, Matuschka FR, Musgrave K, Fischer P, Spielman A. Hosts on Which Nymphal *Ixodes ricinus* Most Abundantly Feed. The American Journal of Tropical Medicine and Hygiene. 1991 Jan;44(1):100–7.
160. L'Hostis M, Dumon H, Fusade A, Lazareff S, Gorenflot A. Seasonal incidence of *Ixodes ricinus* ticks (*Acari: ixodidae*) on rodents in western France. Exp Appl Acarol. 1996 Jul;20(7):359–68.
161. Mihalca AD, Dumitrache MO, Sándor AD, Magdaş C, Oltean M, Györke A, et al. Tick parasites of rodents in Romania: host preferences, community structure and geographical distribution. Parasites Vectors. 2012 Dec;5(1):266.
162. Dizij A, Kurtenbach K. *Clethrionomys glareolus*, but not *Apodemus flavicollis*, acquires resistance to *lxodes ricinus* L, the main European vector of *Borrelia burgdorferi*. Parasite Immunology. 1995 Apr;17(4):177–83.
163. Randolph SE. Density-dependent acquired resistance to ticks in natural hosts, independent of concurrent infection with *Babesia microti*. Parasitology. 1994 May;108(4):413–9.
164. Hughes VL, Randolph SE. Testosterone depresses innate and aquired resistance to ticks in natural rodent hosts: a force for aggregated distributions of parasites. Parasitology. 2001 Feb;87(1):49–54.
165. Talleklint L, Jaenson TGT. Infestation of mammals by *Ixodes ricinus* ticks (*Acari: Ixodidae*) in south-central Sweden. Exp Appl Acarol. 1997;21(12):755–71.
166. Hanincová K, Schäfer SM, Etti S, Sewell HS, Taragelová V, Ziak D, et al. Association of *Borrelia afzelii* with rodents in Europe. Parasitology. 2003 Jan;126(1):11–20.
167. Michalik J, Hofman T, Buczek A, Skoracki M, Sikora B. *Borrelia burgdorferi* s.l. in *Ixodes ricinus* (*Acari: Ixodidae*) Ticks Collected from Vegetation and Small Rodents in Recreational Areas of the City of Poznań. J Med Entomol. 2003 Sep;40(5):690–7.
168. Stanko M, Krasnov BR, Miklisova D, Morand S. Simple epidemiological model predicts the relationships between prevalence and abundance in ixodid ticks. Parasitology. 2007 Jan;134(1):59–68.
169. Kiffner C, Vor T, Hagedorn P, Niedrig M, Rühe F. Factors affecting patterns of tick parasitism on forest rodents in tick-borne encephalitis risk areas, Germany. Parasitol Res. 2011 Feb;108(2):323–35.

170. Boyard C, Vourc'h G, Barnouin J. The relationships between *Ixodes ricinus* and small mammal species at the woodland-pasture interface. Exp Appl Acarol. 2008 Jan;44(1):61–76.
171. Pichon B, Mousson L, Figureau C, Rodhain F, Perez-Eid C. Density of deer in relation to the prevalence of *Borrelia burgdorferi* s.1. in *Ixodes ricinus* nymphs in Rambouillet forest, France. Exp Appl Acarol. 1999;23(3):267–75.
172. Gray JS, Kahl O, Janetzki C, Stein J. Studies on the Ecology of Lyme Disease in a Deer Forest in County Galway, Ireland. Journal of Medical Entomology. 1992 Nov;29(6): 915–20.
173. Tagliapietra V, Rosà R, Arnoldi D, Cagnacci F, Capelli G, Montarsi F, et al. Saturation deficit and deer density affect questing activity and local abundance of *Ixodes ricinus* (*Acari, Ixodidae*) in Italy. Veterinary Parasitology. 2011 Dec;183(1–2):114–24.
174. Gilbert L, Maffey GL, Ramsay SL, Hester AJ. The effect of deer management on the abundance of *Ixodes ricinus* in Scotland. Ecological Applications. 2012 Mar;22(2):658–67.
175. Ruiz-Fons F, Fernández-de-Mera IG, Acevedo P, Gortázar C, de la Fuente J. Factors Driving the Abundance of *Ixodes ricinus* Ticks and the Prevalence of Zoonotic *I. ricinus*-Borne Pathogens in Natural Foci. Appl Environ Microbiol. 2012 Apr;78(8): 2669–76.
176. James MC, Bowman AS, Forbes KJ, Lewis F, Mcleod JE, Gilbert L. Environmental determinants of *Ixodes ricinus* ticks and the incidence of *Borrelia burgdorferi* sensu lato, the agent of Lyme borreliosis, in Scotland. Parasitology. 2013 Feb;140(2):237–46.
177. Hofmeester TR, Sprong H, Jansen PA, Prins HHT, Van Wieren SE. Deer presence rather than abundance determines the population density of the sheep tick, *Ixodes ricinus*, in Dutch forests. Parasites Vectors. 2017 Dec;10(1):433.
178. Dobson ADM, Randolph SE. Modelling the effects of recent changes in climate, host density and acaricide treatments on population dynamics of *Ixodes ricinus* in the UK: *Ixodes ricinus* population change model. Journal of Applied Ecology. 2011 Aug;48(4): 1029–37.
179. Handeland K, Qviller L, Vikøren T, Viljugrein H, Lillehaug A, Davidson RK. *Ixodes ricinus* infestation in free-ranging cervids in Norway – A study based upon ear examinations of hunted animals. Veterinary Parasitology. 2013 Jul;195(1–2):142–9.
180. Vor T, Kiffner C, Hagedorn P, Niedrig M, Rühe F. Tick burden on European roe deer (*Capreolus capreolus*). Exp Appl Acarol. 2010 Aug;51(4):405–17.
181. Kiffner C, Lödige C, Alings M, Vor T, Rühe F. Abundance estimation of *Ixodes* ticks (*Acari: Ixodidae*) on roe deer (*Capreolus capreolus*). Exp Appl Acarol. 2010 Sep;52(1): 73–84.
182. Król N, Chitimia-Dobler L, Dobler G, Karliuk Y, Birka S, Obiegala A, et al. Tick burden on European roe deer (*Capreolus capreolus*) from Saxony, Germany, and detection of tick-borne encephalitis virus in attached ticks. Parasitol Res. 2020 Apr;119(4): 1387–92.
183. Opalińska P, Wierzbicka A, Asman M, Rączka G, Dyderski MK, Nowak-Chmura M. Fivefold higher abundance of ticks (*Acari: Ixodidae*) on the European roe deer (*Capreolus capreolus* L.) forest than field ecotypes. Sci Rep. 2021 May;11(1):10649.
184. Rizzoli A, Hauffe HC, Tagliapietra V, Neteler M, Rosà R. Forest structure and roe deer abundance predict tick-borne encephalitis risk in Italy. PLoS ONE. 2009 Feb;4(2): e4336.

185. Carpi G, Cagnacci F, Neteler M, Rizzoli A. Tick infestation on roe deer in relation to geographic and remotely sensed climatic variables in a tick-borne encephalitis endemic area. Epidemiol Infect. 2008 Oct;136(10):1416–24.
186. Knap N, Avšič-Županc T. Correlation of TBE incidence with red deer and roe deer abundance in Slovenia. PLoS ONE. 2013 Jun;8(6):e66380.
187. Kriz B, Daniel M, Benes C, Maly M. The role of game (wild boar and roe deer) in the spread of tick-borne encephalitis in the Czech Republic. Vector-Borne and Zoonotic Diseases. 2014 Nov;14(11):801–7.
188. Cagnacci F, Bolzoni L, Rosà R, Carpi G, Hauffe HC, Valent M, et al. Effects of deer density on tick infestation of rodents and the hazard of tick-borne encephalitis. I: Empirical assessment. International Journal for Parasitology. 2012 Apr;42(4):365–72.
189. Perkins SE, Cattadori IM, Tagliapietra V, Rizzoli AP, Hudson PJ. Localized deer absence leads to tick amplification. Ecology. 2006 Aug;87(8):1981–6.
190. Paulsen KM, das Neves CG, Granquist EG, Madslien K, Stuen S, Pedersen BN, et al. Cervids as sentinel-species for tick-borne encephalitis virus in Norway – A serological study. Zoonoses and Public Health. 2020 Jun;67(4):342–51.
191. Ytrehus B, Vainio K, Dudman SG, Gilray J, Willoughby K. Tick-Borne Encephalitis Virus and Louping-Ill Virus May Co-Circulate in Southern Norway. Vector-Borne and Zoonotic Diseases. 2013 Oct;13(10):762–8.
192. Duscher GG, Wetscher M, Baumgartner R, Walder G. Roe deer sera used for TBE surveillance in Austria. Ticks and Tick-borne Diseases. 2015 Jun;6(4):489–93.
193. Potkonjak A, Petrović T, Ristanović E, Lalić I, Vračar V, Savić S, et al. Molecular Detection and Serological Evidence of Tick-Borne Encephalitis Virus in Serbia. Vector-Borne and Zoonotic Diseases. 2017 Dec;17(12):813–20.
194. Andersen NS, Larsen SL, Olesen CR, Stiasny K, Kolmos HJ, Jensen PM, et al. Continued expansion of tick-borne pathogens: Tick-borne encephalitis virus complex and *Anaplasma phagocytophilum* in Denmark. Ticks and Tick-borne Diseases. 2019 Jan;10(1):115–23.
195. Skarphédinsson S, Jensen PM, Kristiansen K. Survey of Tick-borne Infections in Denmark. Emerg Infect Dis. 2005 Jul;11(7):1055–61.
196. Cisak E, Wójcik-Fatla A, Sroka J, Zając V, Bilska-Zając E, Chmurzyńska E, et al. Prevalence of Tick-Borne Encephalitis Virus Antibodies in Domestic and Game Animals from Eastern Poland. Bulletin of the Veterinary Institute in Pulawy. 2012 Sep; 56(3):275–8.
197. Linden A, Wirtgen M, Nahayo A, Heyman P, Niedrig M, Schulze Y. Tick-borne encephalitis virus antibodies in wild cervids in Belgium. Veterinary Record. 2012 Jan; 170(4):108–108.
198. Grassi L, Drigo M, Zelená H, Pasotto D, Cassini R, Mondin A, et al. Wild ungulates as sentinels of flaviviruses and tick-borne zoonotic pathogen circulation: an Italian perspective. BMC Vet Res. 2023 Sep;19(1):155.
199. Gomez Martinez C. Role of cervids and wild boar on the presence of tick-borne encephalitis virus in Sweden. [Internet]. Master Thesis in Biology at the Department of Wildlife, Fish, and Environmental Studies, Sweden, Umea; 2014 [Internet]. [cited 27 Mar 2024]. Available from: https://brage.inn.no/inn-xmlui/bitstream/handle/11250/285492/Carmelo%20Martinez.pdf?sequence=1&isAllowed=y
200. Topp AK, Springer A, Mischke R, Rieder J, Feige K, Ganter M, et al. Seroprevalence of tick-borne encephalitis virus in wild and domestic animals in northern Germany. Ticks and Tick-borne Diseases. 2023 Nov;14(6):102220.

201. Frimmel S, Krienke A, Riebold D, Loebermann M, Littmann M, Fiedler K, et al. Tick-borne encephalitis virus habitats in North East Germany: reemergence of TBEV in ticks after 15 years of inactivity. BioMed Research International. 2014;2014:1–5.
202. Balling A, Plessow U, Beer M, Pfeffer M. Prevalence of antibodies against tick-borne encephalitis virus in wild game from Saxony, Germany. Ticks and Tick-borne Diseases. 2014 Oct;5(6):805–9.
203. Kiffner C, Vor T, Hagedorn P, Niedrig M, Rühe F. Determinants of tick-borne encephalitis virus antibody presence in roe deer (*Capreolus capreolus*) sera. Medical Vet Entomology. 2012 Mar;26(1):18–25.
204. Tonteri E, Jokelainen P, Matala J, Pusenius J, Vapalahti O. Serological evidence of tick-borne encephalitis virus infection in moose and deer in Finland: sentinels for virus circulation. Parasites Vectors. 2016 Dec;9(1):54.
205. Jamsransuren D, Yoshii K, Kariwa H, Asakawa M, Okuda K, Fujii K, et al. Epidemiological survey of tick-borne encephalitis virus infection in wild animals on Hokkaido and Honshu islands, Japan. [Internet]. Faculty of Veterinary Medicine, Hokkaido University; 2019 [cited 2024 Apr 2]. Available from: https://doi.org/10.14943/jjvr.67.2.163
206. Krzysiak MK, Anusz K, Konieczny A, Rola J, Salat J, Strakova P, et al. The European bison (*Bison bonasus*) as an indicatory species for the circulation of tick-borne encephalitis virus (TBEV) in natural foci in Poland. Ticks and Tick-borne Diseases. 2021 Nov;12(6):101799.
207. Jemeršić L, Dežđek D, Brnić D, Prpić J, Janicki Z, Keros T, et al. Detection and genetic characterization of tick-borne encephalitis virus (TBEV) derived from ticks removed from red foxes (*Vulpes vulpes*) and isolated from spleen samples of red deer (*Cervus elaphus*) in Croatia. Ticks and Tick-borne Diseases. 2014 Feb;5(1):7–13.
208. Da Rold G, Obber F, Monne I, Milani A, Ravagnan S, Toniolo F, et al. Clinical Tick-Borne Encephalitis in a Roe Deer (*Capreolus capreolus* L.). Viruses. 2022 Jan;14(2):300.
209. Roelandt S, Suin V, Stede YVD, Lamoral S, Marche S, Tignon M, et al. First TBEV serological screening in Flemish wild boar. Infection Ecology & Epidemiology. 2016 Jan;6(1):31099.
210. Adjadj NR, Vervaeke M, Sohier C, Cargnel M, De Regge N. Tick-borne encephalitis virus prevalence in sheep, wild boar and ticks in Belgium. Viruses. 2022 Oct;14(11):2362.
211. Van der Poel WHM, Van der Heide R, Bakker D, De Looff M, De Jong J, Van Manen N, et al. Attempt to detect evidence for tick-borne encephalitis virus in ticks and mammalian wildlife in the Netherlands. Vector-Borne and Zoonotic Diseases. 2005 Mar;5(1):58–64.
212. Bournez L, Umhang G, Faure E, Boucher JM, Boué F, Jourdain E, et al. Exposure of wild ungulates to the Usutu and Tick-Borne Encephalitis Viruses in France in 2009–2014: Evidence of undetected flavivirus circulation a decade Ago. Viruses. 2019 Dec;12(1):10.
213. Lorusso V, Lia RP, Dantas-Torres F, Mallia E, Ravagnan S, Capelli G, et al. Ixodid ticks of road-killed wildlife species in southern Italy: new tick-host associations and locality records. Exp Appl Acarol. 2011 Nov;55(3):293–300.
214. Sobrino R, Millán J, Oleaga Á, Gortázar C, de la Fuente J, Ruiz-Fons F. Ecological preferences of exophilic and endophilic ticks (*Acari: Ixodidae*) parasitizing wild carnivores in the Iberian Peninsula. Veterinary Parasitology. 2012 Mar;184(2–4):248–57.
215. Meyer-Kayser E, Hoffmann L, Silaghi C, Passos L, Mahling M, Pfister K. Tick infestation of foxes in Thuringia with special focus on foxes with scabies. Ticks Tick Borne Dis. 2012 Sep;3(4):232-9.

216. Sréter T, Széll Z, Varga I. Ectoparasite infestations of red foxes (*Vulpes vulpes*) in Hungary. Veterinary Parasitology. 2003 Aug;115(4):349–54.
217. Takumi K, Sprong H, Hofmeester TR. Impact of vertebrate communities on *Ixodes ricinus*-borne disease risk in forest areas. Parasites Vectors. 2019 Dec;12(1):434.
218. Kiffner C, Zucchini W, Schomaker P, Vor T, Hagedorn P, Niedrig M, et al. Determinants of tick-borne encephalitis in counties of southern Germany, 2001-2008. Int J Health Geogr. 2010;9(1):42.
219. Haemig PD, Lithner S, Sjöstedt de luna S, Lundkvist Å, Waldenström J, Hansson L, et al. Red fox and tick-borne encephalitis (TBE) in humans: Can predators influence public health? Scandinavian Journal of Infectious Diseases. 2008 Jan;40(6–7):527–32.
220. Haut M, Girl P, Oswald B, Romig T, Obiegala A, Dobler G, et al. The Red Fox (*Vulpes vulpes*) as Sentinel for Tick-Borne Encephalitis Virus in Endemic and Non-Endemic Areas. Microorganisms. 2020 Nov;8(11):1817.
221. Rieger MA, Nübling M, Müller W, Hasselhorn HM, Hofmann F. Foxes as indicators for TBE endemicity – a comparative serological investigation. Zentralblatt für Bakteriologie. 1999 Dec;289(5–7):610–8.
222. Wurm R, Dobler G, Peters M, Kiessig ST. Serological Investigations of Red Foxes (*Vulpes vulpes* L.) for Determination of the Spread of Tick-borne Encephalitis in Northrhine-Westphalia. Journal of Veterinary Medicine, Series B. 2000 Sep;47(7): 503–9.
223. Gray J, Kahl O, Zintl A. What do we still need to know about *Ixodes ricinus*? Ticks and Tick-borne Diseases. 2021 May;12(3):101682.
224. Gray JS. The ecology of Lyme borreliosis vector ticks. In: Bruin J, Van der Geest LPS, Sabelis MW, editors. Ecology and Evolution of the Acari. [Internet]. Dordrecht: Springer Netherlands; 1999 [cited 2024 Mar 31]. p. 269-76 Available from: https://link.springer.com/10.1007/978-94-017-1343-6_18
225. Abdul-Amir IM, Gray JS. Resistance of sheep to laboratory infestations of the tick, *Ixodes ricinus*. Research in Veterinary Science. 1987 Sep;43(2):266–7.
226. Gassner F, Verbaarschot P, Smallegange RC, Spitzen J, Van Wieren SE, Takken W. Variations in *Ixodes ricinus* Density and *Borrelia* Infections Associated with Cattle Introduced into a Woodland in The Netherlands. Appl Environ Microbiol. 2008 Dec;74(23):7138–44.
227. Van Wieren SE, Hofmeester TR. 6. The role of large herbivores in *Ixodes ricinus* and *Borrelia burgdorferi* s.l. dynamics. In: Braks MAH, Van Wieren SE, Takken W, Sprong H, editors. Ecology and prevention of Lyme borreliosis [Internet]. Brill | Wageningen Academic; 2016 [cited 2024 Mar 31]. p. 75–89. Available from: https://brill.com/view/book/9789086868384/BP000009.xml
228. Klaus C, Beer M, Saier R, Schau U, Moog U, Hoffmann B, et al. Goats and sheep as sentinels for tick-borne encephalitis (TBE) virus – Epidemiological studies in areas endemic and non-endemic for TBE virus in Germany. Ticks and Tick-borne Diseases. 2012 Feb;3(1):27–37.
229. Grandi G, Aspán A, Pihl J, Gustafsson K, Engström F, Jinnerot T, et al. Detection of Tick-Borne Pathogens in Lambs Undergoing Prophylactic Treatment Against Ticks on Two Swedish Farms. Front Vet Sci. 2018 Apr;16;5:72.
230. Šikutová S, Hornok S, Hubálek Z, Doležálková I, Juřicová Z, Rudolf I. Serological survey of domestic animals for tick-borne encephalitis and Bhanja viruses in northeastern Hungary. Veterinary Microbiology. 2009 Mar;135(3–4):267–71.

231. Juceviciene A, Zygutiene M, Leinikki P, Brummer-Korvenkontio H, Salminen M, Han X, et al. Tick-borne encephalitis virus infections in Lithuanian domestic animals and ticks. Scandinavian Journal of Infectious Diseases. 2005 Jan;37(10):742–6.
232. Hubálek Z, Mitterpák J, Prokopic J, Juricová Z, Kilík J. A serological survey for Bhanja and tick-borne encephalitis viruses in sheep of eastern Slovakia. Folia Parasitol (Praha). 1985;32(3):279–83.
233. Casati Pagani S, Frigerio Malossa S, Klaus C, Hoffmann D, Beretta O, Bomio-Pacciorini N, et al. First detection of TBE virus in ticks and sero-reactivity in goats in a non-endemic region in the southern part of Switzerland (Canton of Ticino). Ticks and Tick-borne Diseases. 2019 Jun;10(4):868–74.
234. Rieille N, Klaus C, Hoffmann D, Péter O, Voordouw MJ. Goats as sentinel hosts for the detection of tick-borne encephalitis risk areas in the Canton of Valais, Switzerland. BMC Vet Res. 2017 Dec;13(1):217.
235. Klaus C, Ziegler U, Kalthoff D, Hoffmann B, Beer M. Tick-borne encephalitis virus (TBEV) – findings on cross reactivity and longevity of TBEV antibodies in animal sera. BMC Vet Res. 2014;10(1):78.
236. Stefanoff P, Siennicka J, Kaba J, Nowicki M, Ferenczi E, Gut W. Identification of new endemic tick-borne encephalitis foci in Poland – a pilot seroprevalence study in selected regions. International Journal of Medical Microbiology. 2008 Sep;298:102–7.
237. Holzmann H, Aberle SW, Stiasny K, Werner P, Mischak A, Zainer B, et al. Tick-borne Encephalitis from Eating Goat Cheese in a Mountain Region of Austria. Emerg Infect Dis. 2009 Oct;15(10):1671–3.
238. Ilic M, Barbic L, Bogdanic M, Tabain I, Savic V, Kosanovic Licina ML, et al. Tick-borne encephalitis outbreak following raw goat milk consumption in a new micro-location, Croatia, June 2019. Ticks and Tick-borne Diseases. 2020 Nov;11(6):101513.
239. Alfano N, Tagliapietra V, Rosso F, Ziegler U, Arnoldi D, Rizzoli A. Tick-borne encephalitis foci in northeast Italy revealed by combined virus detection in ticks, serosurvey on goats and human cases. Emerging Microbes & Infections. 2020 Jan;9(1):474–84.
240. Paulsen KM, Stuen S, das Neves CG, Suhel F, Gurung D, Soleng A, et al. Tick-borne encephalitis virus in cows and unpasteurized cow milk from Norway. Zoonoses and Public Health. 2019 Mar;66(2):216–22.
241. Roelandt S, Suin V, Riocreux F, Lamoral S, Van der Heyden S, Van der Stede Y, et al. Autochthonous Tick-Borne Encephalitis Virus-Seropositive Cattle in Belgium: A Risk-Based Targeted Serological Survey. Vector-Borne and Zoonotic Diseases. 2014 Sep;14(9):640–7.
242. Omazic A, Wallenhammar A, Lahti E, Asghar N, Hanberger A, Hjertqvist M, et al. Dairy milk from cow and goat as a sentinel for tick-borne encephalitis virus surveillance. Comparative Immunology, Microbiology and Infectious Diseases. 2023 Apr; 95:101958.
243. Cisak E, Wójcik-Fatla A, Zajac V, Sroka J, Buczek A, Dutkiewicz J. Prevalence of tick-borne encephalitis virus (TBEV) in samples of raw milk taken randomly from cows, goats and sheep in Eastern Poland. Annals of Agricultural and Environmental Medicine. 2010;17(2):283-286.
244. Pautienius A, Dudas G, Simkute E, Grigas J, Zakiene I, Paulauskas A, et al. Bulk Milk Tank Samples Are Suitable to Assess Circulation of Tick-Borne Encephalitis Virus in High Endemic Areas. Viruses. 2021;13(9):1772.
245. Pautienius A, Armonaite A, Simkute E, Zagrabskaite R, Buitkuviene J, Alpizar-Jara R, et al. Cross-Sectional Study on the Prevalence and Factors Influencing Occurrence of Tick-Borne Encephalitis in Horses in Lithuania. Pathogens. 2021;10(2):140.

246. Rushton JO, Lecollinet S, Hubálek Z, Svobodová P, Lussy H, Nowotny N. Tick-borne Encephalitis Virus in Horses, Austria, 2011. Emerg Infect Dis. 2013 Apr;19(4):635–7.
247. Klaus C, Hörügel U, Hoffmann B, Beer M. Tick-borne encephalitis virus (TBEV) infection in horses: Clinical and laboratory findings and epidemiological investigations. Veterinary Microbiology. 2013 May;163(3–4):368–72.
248. Janitza-Futterer D. Serological studies on the endemic situation of infection with TBE virus in a horse and dog population in southern Baden. Doctoral Thesis. Munich: Ludwig-Maximilians-Universität München. 2003.
249. Müller K, König M, Thiel HJ. Tick-borne encephalitis (TBE) with special emphasis on infection in horses. Dtsch Tierarztl Wochenschr. 2006 Apr;113(4):147–51.
250. Gothe LMR, Ganzenberg S, Ziegler U, Obiegala A, Lohmann KL, Sieg M, et al. Horses as Sentinels for the Circulation of Flaviviruses in Eastern–Central Germany. Viruses. 2023 Apr;15(5):1108.
251. Csank T, Drzewnioková P, Korytár Ľ, Major P, Gyuranecz M, Pistl J, et al. A Serosurvey of Flavivirus Infection in Horses and Birds in Slovakia. Vector-Borne and Zoonotic Diseases. 2018 Apr;18(4):206–13.
252. Camino E, Schmid S, Weber F, Pozo P, de Juan L, König M, et al. Detection of antibodies against tick-borne encephalitis flaviviruses in breeding and sport horses from Spain. Ticks and Tick-borne Diseases. 2020 Sep;11(5):101487.
253. Conze TM, Bagó Z, Revilla-Fernández S, Schlegel J, Goehring LS, Matiasek K. Tick-Borne Encephalitis Virus (TBEV) Infection in Two Horses. Viruses. 2021 Sep;13(9): 1775.
254. De Heus P, Bagó Z, Weidinger P, Lale D, Trachsel DS, Revilla-Fernández S, et al. Severe Neurologic Disease in a Horse Caused by Tick-Borne Encephalitis Virus, Austria, 2021. Viruses. 2023 Sep;15(10):2022.
255. Magouras I, Schoster A, Fouché N, Gerber V, Groschup MH, Ziegler U, et al. Neurological disease suspected to be caused by tick-borne encephalitis virus infection in 6 horses in Switzerland. Veterinary Internal Medicine. 2022 Nov;36(6):2254–62.
256. Fouché N, Oesch S, Ziegler U, Gerber V. Clinical Presentation and Laboratory Diagnostic Work-Up of a Horse with Tick-Borne Encephalitis in Switzerland. Viruses. 2021 Jul;13(8):1474.
257. Spitmann N, Wiesner L, Boelke M, Schulz C, Mischke R, Baechlein C, et al. Validation of the luciferase immunoprecipitation system based antibody detection assay for TBE virus in dogs and cats. Tierarztl Prax Ausg K Kleinertiere Heimtiere 2021;49(01):71-72.
258. Riederer SA. A study on the prevalence of the antibodies against the tick-borne encephalitis virus in dogs and cats in Bavaria. Doctoral Thesis, University of Leipzig, Germany. 2021. p. 70.
259. Lommano E, Dvořák C, Vallotton L, Jenni L, Gern L. Tick-borne pathogens in ticks collected from breeding and migratory birds in Switzerland. Ticks and Tick-borne Diseases. 2014 Oct;5(6):871–82.
260. Wilhelmsson P, Jaenson TGT, Olsen B, Waldenström J, Lindgren PE. Migratory birds as disseminators of ticks and the tick-borne pathogens *Borrelia* bacteria and tick-borne encephalitis (TBE) virus: a seasonal study at Ottenby Bird Observatory in South-eastern Sweden. Parasites Vectors. 2020 Dec;13(1):607.
261. Kazarina A, Japiņa K, Keišs O, Salmane I, Bandere D, Capligina V, et al. Detection of tick-borne encephalitis virus in *I. ricinus* ticks collected from autumn migratory birds in Latvia. Ticks and Tick-borne Diseases. 2015 Mar;6(2):178–80.

262. Geller J, Nazarova L, Katargina O, Leivits A, Järvekülg L, Golovljova I. Tick-Borne Pathogens in Ticks Feeding on Migratory Passerines in Western Part of Estonia. Vector-Borne and Zoonotic Diseases. 2013 Jul;13(7):443–8.
263. Movila A, Alekseev AN, Dubinina HV, Toderas I. Detection of tick-borne pathogens in ticks from migratory birds in the Baltic region of Russia. Medical Vet Entomology. 2013 Mar;27(1):113–7.
264. Waldenström J, Lundkvist Å, Falk KI, Garpmo U, Bergström S, Lindegren G, et al. Migrating Birds and Tickborne Encephalitis Virus. Emerg Infect Dis. 2007 Aug;13(8): 1215–8.
265. Burri C, Bastic V, Maeder G, Patalas E, Gern L. Microclimate and the Zoonotic Cycle of Tick-Borne Encephalitis Virus in Switzerland. J Med Entomol. 2011 May;48(3): 615–27.
266. Korobitsyn IG, Moskvitina NS, Tyutenkov OY, Gashkov SI, Kononova YV, Moskvitin SS, et al. Detection of tick-borne pathogens in wild birds and their ticks in Western Siberia and high level of their mismatch. Folia Parasitol (Praha). 2021 Nov;68:2021.024.
267. Ličková M, Fumačová Havlíková S, Slávikova M, Klempa B. Alimentary Infections by Tick-Borne Encephalitis Virus. Viruses. 2021 Dec;14(1):56.
268. Martello E, Gillingham EL, Phalkey R, Vardavas C, Nikitara K, Bakonyi T, et al. Systematic review on the non-vectorial transmission of Tick-borne encephalitis virus (TBEv). Ticks and Tick-borne Diseases. 2022 Nov;13(6):102028.
269. Divé I, Veje M, Dobler G, Bergström T, Buxmann H, Paul B, et al. Tick-borne encephalitis virus (TBEV) infection in pregnancy: Absence of virus transmission to the fetuses despite severe maternal disease – A case study. Ticks and Tick-borne Diseases. 2020 Sep;11(5):101491.
270. Lipowski D, Popiel M, Perlejewski K, Nakamura S, Bukowska-Ośko I, Rzadkiewicz E, et al. A Cluster of Fatal Tick-borne Encephalitis Virus Infection in Organ Transplant Setting. The Journal of Infectious Diseases. 2017 Mar;215(6):896–901.
271. Wahlberg P, Saikku P, Brummer-Korvenkontio M. Tick-borne viral encephalitis in Finland. The clinical features of Kumlinge disease during 1959–1987. Journal of Internal Medicine. 1989 Mar;225(3):173–7.
272. Bodemann HH, Pausch J, Schmitz H, Hoppe-Seyler G. Tick-born encephalitis (ESME) as laboratory infection. Med Welt. 1977 Nov;28(44):1779–81.
273. Avšič-Županc T, Poljak M, Matičič M, Radšel-Medveščček A, LeDuc JW, Stiasny K, et al. Laboratory acquired tick-borne meningoencephalitis: characterisation of virus strains. Clinical and Diagnostic Virology. 1995 Jul;4(1):51–9.
274. Taba P, Schmutzhard E, Forsberg P, Lutsar I, Ljøstad U, Mygland Å, et al. EAN consensus review on prevention, diagnosis and management of tick-borne encephalitis. Euro J of Neurology. 2017 Oct;24(10):1214.
275. Kaiser R. Tick-borne encephalitis. Infect Dis Clin North Am. 2008 Sep;22(3):561–75, x.
276. Heinze DM, Gould EA, Forrester NL. Revisiting the Clinal Concept of Evolution and Dispersal for the Tick-Borne Flaviviruses by Using Phylogenetic and Biogeographic Analyses. J Virol. 2012 Aug;86(16):8663–71.
277. Kaiser R. The clinical and epidemiological profile of tick-borne encephalitis in southern Germany 1994–98. Brain. 1999 Nov;122(11):2067–78.
278. Kaiser R. Tick-borne encephalitis: Clinical findings and prognosis in adults. Wien Med Wochenschr. 2012 Jun;162(11–12):239–43.

279. Parfut A, Laugel E, Baer S, Gonzalez G, Hansmann Y, Wendling MJ, et al. Tick-borne encephalitis in pediatrics: An often overlooked diagnosis. Infectious Diseases Now. 2023 Mar;53(2):104645.
280. Mickienė A, Laiškonis A, Günther G, Vene S, Lundkvist Å, Lindquist L. Tickborne Encephalitis in an Area of High Endemicity in Lithuania: Disease Severity and Long-Term Prognosis. Clin Infect Dis. 2002 Sep;35(6):650–8.
281. Fauser S, Stich O, Rauer S. Unusual case of tick-borne encephalitis with isolated myeloradiculitis. Journal of Neurology, Neurosurgery & Psychiatry. 2007 Aug; 78(8):909-10.
282. Neumann B, Schulte-Mattler W, Brix S, Pöschl P, Jilg W, Bogdahn U, et al. Autonomic and peripheral nervous system function in acute tick-borne encephalitis. Brain and Behavior. 2016 Aug;6(8):e00485.
283. Jereb M, Muzlovic I, Avsic-Zupanc T, Karner P. Severe tick-borne encephalitis in Slovenia: epidemiological, clinical and laboratory findings. Wien Klin Wochenschr. 2002 Jul;114(13–14):623–6.
284. Kleiter I, Steinbrecher A, Flügel D, Bogdahn U, Schulte-Mattler W. Autonomic involvement in tick-borne encephalitis (TBE): report of five cases. Eur J Med Res. 2006 Jun; 11(6):261–5.
285. Gelpi E, Preusser M, Garzuly F, Holzmann H, Heinz FX, Budka H. Visualization of Central European Tick-Borne Encephalitis Infection in Fatal Human Cases. J Neuropathol Exp Neurol. 2005 Jun;64(6):506–12.
286. Park M. Acute anterior radiculitis associated with West Nile virus infection. Journal of Neurology, Neurosurgery & Psychiatry. 2003 Jun;74(6):823–5.
287. De Vries L, Harding AT. Mechanisms of Neuroinvasion and Neuropathogenesis by Pathologic Flaviviruses. Viruses. 2023 Jan;15(2):261.
288. Lotrič-Furlan S, Strle F. Thrombocytopenia, Leukopenia and abnormal liver function tests in the initial phase of Tick-borne Encephalitis. Zentralblatt für Bakteriologie. 1995 Apr;282(3):275–8.
289. Misić-Majerus L, Bujić N, Madarić V, Avsić-Zupanc T. Hepatitis caused by tick-borne meningoencephalitis virus (TBEV) – a rare clinical manifestation outside the central nervous system involvement. Acta Med Croatica. 2005;59(4):347–52.
290. Zambito Marsala S, Francavilla E, Gioulis M, Candeago RM, Mondardini V, Gentile M, et al. Isolated polio-like syndrome after tick-borne encephalitis presenting with acute hyperckemia. Neurol Sci. 2012 Jun;33(3):669–72.
291. Aendekerk RPP, Schrivers ANA, Koehler PJ. Tick-borne encephalitis complicated by a polio-like syndrome following a holiday in central Europe. Clinical Neurology and Neurosurgery. 1996 Aug;98(3):262–4.
292. Mickienė A, Vene S, Golovljova I, Laiškonis A, Lindquist L, Plyusnin A, et al. Tick-borne Encephalitis Virus in Lithuania. Eur J Clin Microbiol Infect Dis. 2001 Dec; 20(12):886–8.
293. Panatto D, Domnich A, Amicizia D, Reggio P, Iantomasi R. Vaccination against Tick-Borne Encephalitis (TBE) in Italy: Still a Long Way to Go. Microorganisms. 2022 Feb;10(2):464.
294. Haglund M, Forsgren M, Lindh G, Lindquist L. A 10-Year Follow-Up Study of Tick-Borne Encephalitis in the Stockholm Area and a Review of the Literature: Need for a Vaccination Strategy. Scandinavian Journal of Infectious Diseases. 1996 Jan;28(3): 217–24.

295. Logar M, Bogovič P, Cerar D, Avšič-Županc T, Strle F. Tick-borne encephalitis in Slovenia from 2000 to 2004: Comparison of the course in adult and elderly patients. Wien Klin Wochenschr. 2006 Nov;118(21–22):702–7.
296. Arnez M, Luznik-Bufon T, Avsic-Zupanc T, Ruzic-Sabljic E, Petrovec M, Lotric-Furlan S, et al. Causes of febrile illnesses after a tick bite in Slovenian children: The Pediatric Infectious Disease Journal. 2003 Dec;22(12):1078–83.
297. Hellenbrand W, Kreusch T, Böhmer M, Wagner-Wiening C, Dobler G, Wichmann O, et al. Epidemiology of Tick-Borne Encephalitis (TBE) in Germany, 2001–2018. Pathogens. 2019 Mar;8(2):42.
298. Lenhard T, Ott D, Jakob NJ, Pham M, Bäumer P, Martinez-Torres F, et al. Predictors, Neuroimaging Characteristics and Long-Term Outcome of Severe European Tick-Borne Encephalitis: A Prospective Cohort Study. PLoS ONE. 2016 Apr;11(4):e0154143.
299. Radzišauskienė D, Urbonienė J, Kaubrys G, Andruškevičius S, Jatužis D, Matulytė E, et al. The epidemiology, clinical presentation, and predictors of severe Tick-borne encephalitis in Lithuania, a highly endemic country: A retrospective study of 1040 patients. PLoS ONE. 2020 Nov;15(11):e0241587.
300. Wu J, Zhang L, Wang X. Host Sex Steroids Interact With Virus Infection: New Insights Into Sex Disparity in Infectious Diseases. Front Microbiol. 2021 Nov;12:747347.
301. Soucy G, Boivin G, Labrie F, Rivest S. Estradiol Is Required for a Proper Immune Response to Bacterial and Viral Pathogens in the Female Brain. The Journal of Immunology. 2005 May;174(10):6391–8.
302. Albinsson B, Hoffman T, Kolstad L, Bergström T, Bogdanovic G, Heydecke A, et al. Seroprevalence of tick-borne encephalitis virus and vaccination coverage of tick-borne encephalitis, Sweden, 2018 to 2019. Euro Surveill. 2024 Jan;29(2):2300221.
303. Ackermann-Gäumann R, Eyer C, Vock M, Gowland P, Tinguely C, Leib SL, et al. Prevalence of anti-tick-borne encephalitis virus (TBEV) antibodies in Swiss blood donors in 2014-2015. Blood Transfusion. 2022 Aug;21(2):100–9.
304. Ganbold D, Uudus B, Nyamdavaa N, Chultemsuren Y, Zagd A, Tangad M, et al. Seroprevalence and risk factors of tick-borne encephalitis in Mongolia between 2016 and 2022. Parasite Epidemiology and Control. 2023 Aug;22:e00318.
305. Banović P, Obregón D, Mijatović D, Simin V, Stankov S, Budakov-Obradović Z, et al. Tick-Borne Encephalitis Virus Seropositivity among Tick Infested Individuals in Serbia. Pathogens. 2021 Mar;10(3):301.
306. Bušová A, Dorko E, Feketeová E, Bereš M, Rimárová K, Diabelková J, et al. Serostatus and risk factors of tick-borne encephalitis. Cent Eur J Public Health. 2018 Dec; 26(Supplement):S56–60.
307. Karelis G, Bormane A, Logina I, Lucenko I, Suna N, Krumina A, et al. Tick-borne encephalitis in Latvia 1973–2009: epidemiology, clinical features and sequelae. Euro J of Neurology. 2012 Jan;19(1):62–8.
308. Bogovič P, Stupica D, Rojko T, Lotrič-Furlan S, Avšič-Županc T, Kastrin A, et al. The long-term outcome of tick-borne encephalitis in Central Europe. Ticks and Tick-borne Diseases. 2018 Feb;9(2):369–78.
309. Varis A, Oksi J, Järveläinen H. Central nervous system infection--tick-borne encephalitis, neuroborreliosis or both? Duodecim. 2011;127(1):75–9.
310. Cimperman J, Maraspin V, Lotrič-Furlan S, Ružić-Sabljić E, Avšič-Županc T, Picken RN, et al. Concomitant infection with tick-borne encephalitis virus and *Borrelia burgdorferi* sensu lato in patients with acute meningitis or meningoencephalitis. Infection. 1998 May;26(3):160–4.

311. Logina I, Krumina A, Karelis G, Elsone L, Viksna L, Rozentale B, et al. Clinical features of double infection with tick-borne encephalitis and Lyme borreliosis transmitted by tick bite. Journal of Neurology, Neurosurgery & Psychiatry. 2006 Jul;77(12):1350–3.
312. Dumpis U, Crook D, Oksi J. Tick-Borne Encephalitis. Clin Infect Dis. 1999 Apr;28(4): 882–90.
313. Salat J, Hunady M, Schanilec P, Strakova P, Stefanik M, Svoboda P, et al. Experimental and Natural Infections of Tick-Borne Encephalitis Virus in Dogs. Viruses. 2021 Oct;13(10):2039.
314. Klimeš J, Literák I, Schánilec P, Juřicová Z, Trachta e Silva E. Prevalence of antibodies to tick-borne encephalitis and West Nile flaviviruses and the clinical signs of tick-borne encephalitis in dogs in the Czech Republic. Veterinary Record. 2001 Jan;148(1):17–20.
315. Dawson KLD, Rosato G, Egloff S, Burgener C, Oevermann A, Grest P, et al. Fatal tick-borne encephalitis virus infection in Dalmatian puppy-dogs after putative vector independent transmission. Veterinary Quarterly. 2024 Dec;44(1):1–7.
316. Kirtz G. Tick-borne encephalitis in an Austrian dog population. Doctoral Thesis. University of Veterinary Medicine, Vienna. 1999.
317. Stadtbaumer K, Leschnik MW, Nell B. Tick-borne encephalitis virus as a possible cause of optic neuritis in a dog. Vet Ophthalmol. 2004 Jul;7(4):271–7.
318. Weissenböck H, Suchy A, Holzmann H. Tick-borne encephalitis in dogs: neuropathological findings and distribution of antigen. Acta Neuropathologica. 1998 Apr;95(4): 361–6.
319. Estrada-Peña A, Pfäffle MP, Petney TN. In Estrada-Peña A, Mihalca AD, Petney TN, editors. Ticks of Europe and North Africa. A Guide to Species Identification. Springer International Publishing; 2017. pp. XXI, 404.
320. Sidorenko M, Radzijevskaja J, Mickevičius S, Bratčikovienė N, Paulauskas A. Prevalence of tick-borne encephalitis virus in questing *Dermacentor reticulatus* and *Ixodes ricinus* ticks in Lithuania. Ticks and Tick-borne Diseases. 2021 Jan;12(1):101594.
321. Prusaite J, Mazeikyte R, Pauza D, Pauziene N, Baleisis R, Juskaitis R, Mickus A, Janulaitis Z. Lithuanian fauna. Mammals. Vilnius, Mokslas. 1988.
322. Logminas V, Prusaite J, Virbickas J. Guide of Identification of Lithuanian Vertebral Animals. Vilnius, Mokslas. 1982.
323. Schwaiger M, Cassinotti P. Development of a quantitative real-time RT-PCR assay with internal control for the laboratory detection of tick borne encephalitis virus (TBEV) RNA. Journal of Clinical Virology. 2003 Jul;27(2):136–45.
324. Puchhammer-Stöckl E, Kunz C, Mandl CW, Heinz FX. Identification of tick-borne encephalitis virus ribonucleic acid in tick suspensions and in clinical specimens by a reverse transcription-nested polymerase chain reaction assay. Clinical and Diagnostic Virology. 1995 Dec;4(4):321–6.
325. Süss J, Béziat P, Ramelow C, Kahl O. Tick-Borne Encephalitis Virus (TBEV)-specific RT-PCR for Characterization of Natural Foci of TBE and for Other Applications. Zentralblatt für Bakteriologie. 1997 Jun;286(1):125–38.
326. Davila E, Fernández-Santos NA, Estrada-Franco JG, Wei L, Aguilar-Durán JA, López-López MJ, et al. Domestic Dogs as Sentinels for West Nile Virus but not *Aedes* -borne Flaviviruses, Mexico. Emerg Infect Dis. 2022 May;28(5):1071–4.
327. Saksida A, Duh D, Lotrič-Furlan S, Strle F, Petrovec M, Avšič-Županc T. The importance of tick-borne encephalitis virus RNA detection for early differential diagnosis of tick-borne encephalitis. Journal of Clinical Virology. 2005 Aug;33(4):331–5.

328. Schultze D, Dollenmaier G, Rohner A, Guidi T, Cassinotti P. Benefit of detecting tick-borne encephalitis viremia in the first phase of illness. Journal of Clinical Virology. 2007 Feb;38(2):172–5.
329. Hekrlová A, Kubíček O, Lány P, Rosenbergová K, Schánilec P. Tick-borne encephalitis in dogs: application of "nested real-time RT-PCR" for intravital virus detection. Berl Munch Tierarztl Wochenschr. 2015;128(9–10):397–401.
330. Reiner B, Grasmück S, Steffen F, Djuric N, Schindler T, Müller W, et al. Prevalence of TBE antibodies in serum and CSF of dogs with inflammatory and non-inflammatory CNS disease. International Journal of Medical Microbiology. 2002 Jun;291:234.
331. Levanov L, Vera CP, Vapalahti O. Prevalence estimation of tick-borne encephalitis virus (TBEV) antibodies in dogs from Finland using novel dog anti-TBEV IgG MAb-capture and IgG immunofluorescence assays based on recombinant TBEV subviral particles. Ticks and Tick-borne Diseases. 2016 Jul;7(5):979–82.
332. Lindhe KE, Meldgaard DS, Jensen PM, Houser GA, Berendt M. Prevalence of tick-borne encephalitis virus antibodies in dogs from Denmark. Acta Vet Scand. 2009 Dec;51(1):56.
333. Balling A, Beer M, Gniel D, Pfeffer M. Prevalence of antibodies against Tick-Borne Encephalitis virus in dogs from Saxony, Germany. Berl Munch Tierarztl Wochenschr. 2015;128(7–8):297–303.
334. García-Bocanegra I, Jurado-Tarifa E, Cano-Terriza D, Martínez R, Pérez-Marín JE, Lecollinet S. Exposure to West Nile virus and tick-borne encephalitis virus in dogs in Spain. Transbound Emerg Dis. 2018 Jun;65(3):765–72.
335. Roelandt S, Heyman P, De Filette M, Vene S, Van der Stede Y, Caij AB, et al. Tick-Borne Encephalitis Virus Seropositive Dog Detected in Belgium: Screening of the Canine Population as Sentinels for Public Health. Vector-Borne and Zoonotic Diseases. 2011 Oct;11(10):1371–6.
336. Lindquist L, Vapalahti O. Tick-borne encephalitis. The Lancet. 2008 May;371(9627): 1861–71.
337. Phipps LP, Johnson N. JMM Profile: Tick-borne encephalitis virus. J Med Microbiol. 2022 May;71(5).
338. Gonzalo-Nadal V, Kohl A, Rocchi M, Brennan B, Hughes J, Nichols J, et al. Suspected tick-borne flavivirus meningoencephalomyelitis in dogs from the UK: six cases (2021). J of Small Animal Practice. 2024 Feb;65(2):132–43.
339. Holding M, Dowall S, Hewson R. Detection of tick-borne encephalitis virus in the UK. The Lancet. 2020 Feb;395(10222):411.
340. Sioutas G, Tsakou K, Top C, Jongejan F, Papadopoulos E. First clinical case of tick-borne encephalitis (TBE) in a dog in Greece. Ticks and Tick-borne Diseases. 2023 Nov;14(6):102226.
341. Radzišauskienė D, Žagminas K, Ašoklienė L, Jasionis A, Mameniškienė R, Ambrozaitis A, et al. Epidemiological patterns of tick-borne encephalitis in Lithuania and clinical features in adults in the light of the high incidence in recent years: a retrospective study. Eur J Neurol. 2018 Feb;25(2):268–74.
342. Kleiter I, Jilg W, Bogdahn U, Steinbrecher A. Delayed Humoral Immunity in a Patient with Severe Tick-borne Encephalitis after Complete Active Vaccination. Infection. 2007 Feb;35(1):26–9.
343. Bender A, Jäger G, Scheuerer W, Feddersen B, Kaiser R, Pfister HW. Two severe cases of tick-borne encephalitis despite complete active vaccination the significance of neutralizing antibodies. Journal of Neurology. 2004 Mar;251(3):353–4.

344. Haslwanter D, Blaas D, Heinz FX, Stiasny K. A novel mechanism of antibody-mediated enhancement of flavivirus infection. PLoS Pathog. 2017 Sep;13(9):e1006643.
345. Phillpotts RJ, Stephenson JR, Porterfield JS. Antibody-dependent Enhancement of Tick-borne Encephalitis Virus Infectivity. Journal of General Virology. 1985 Aug; 66(8): 831–7.
346. Nygren T, Pilic A, Böhmer M, Wagner-Wiening C, Wichmann O, Harder T, et al. Tick-Borne Encephalitis Risk Increases with Dog Ownership, Frequent Walks, and Gardening: A Case-Control Study in Germany 2018–2020. Microorganisms. 2022 Mar;10(4):690.
347. Bogovič P, Kastrin A, Lotrič-Furlan S, Ogrinc K, Avšič Županc T, Korva M, et al. Comparison of laboratory and immune characteristics of the initial and second phase of tick-borne encephalitis. Emerging Microbes & Infections. 2022 Dec;11(1):1647–56.
348. Kaiser R, Holzmann H. Laboratory Findings in Tick-Borne Encephalitis - Correlation with Clinical Outcome. Infection. 2000 Apr;28(2):78–84.
349. Jones N, Sperl W, Koch J, Holzmann H, Radauer W. Tick-borne encephalitis in a 17-day-old newborn resulting in severe neurologic impairement. The Pediatric Infectious Disease Journal. 2007 Feb;26(2):185–6.
350. Steffen R. Tick-borne encephalitis (TBE) in children in Europe: Epidemiology, clinical outcome and comparison of vaccination recommendations. Ticks and Tick-borne Diseases. 2019 Jan;10(1):100–10.
351. Salat J, Hunady M, Svoboda P, Strelcova L, Strakova P, Fortova A, et al. Efficacy and immunogenicity of a veterinary vaccine candidate against tick-borne encephalitis in dogs. Vaccine. 2023 Oct;41(42):6150–5.
352. Kosina P, Plisek S, Krausova J, Kracmarova R. Tick-borne encephalitis virus – a rare cause of encephalitis in infants. Wien Klin Wochenschr. 2008 Nov;120(21–22):710–1.
353. Krbková L, Čapovová I, Homola L, Lindušková J, Salát J, Růžek D. Tick-Borne Encephalitis in an 8.5-Month-Old Boy Suspected of Febrile Seizures. Microorganisms. 2021 Jul;9(7):1425.
354. Iff T, Meier R, Olah E, Schneider JFL, Tibussek D, Berger C. Tick-borne meningo-encephalitis in a 6-week-old infant. Eur J Pediatr. 2005 Dec;164(12):787–8.
355. Raja H, Starr MR, Bakri SJ. Ocular manifestations of tick-borne diseases. Survey of Ophthalmology. 2016 Nov;61(6):726–44.
356. Voulgari N, Blanc CM, Guido V, Rossi DC, Guex-Crosier Y, Hoogewoud F. Tick-borne encephalitis related uveitis: a case report. BMC Ophthalmol. 2021 Dec;21(1):315.
357. Ness T, Hengel H. Adverse ocular effects of vaccination. Ophthalmologe. 2016 Jul; 113(7):615–22.
358. Ness T, Boehringer D, Heinzelmann S. Intermediate uveitis: pattern of etiology, complications, treatment and outcome in a tertiary academic center. Orphanet J Rare Dis. 2017 Dec;12(1):81.
359. Doser AK, Hartmann K, Fleisch F, Kuhn M. Suspected neurological side-effects of tick-borne meningoencephalitis vaccination: experiences of the Swiss Adverse Drug Reaction Reporting Center. Praxis (Bern 1994). 2002 Jan;91(5):159-62.
360. Zambito Marsala S, Pistacchi M, Gioulis M, Mel R, Marchini C, Francavilla E. Neurological complications of tick borne encephalitis: the experience of 89 patients studied and literature review. Neurol Sci. 2014 Jan;35(1):15–21.
361. Boelke M, Bestehorn M, Marchwald B, Kubinski M, Liebig K, Glanz J, et al. First Isolation and Phylogenetic Analyses of Tick-Borne Encephalitis Virus in Lower Saxony, Germany. Viruses. 2019 May;11(5):462.

362. Paulsen KM, Pedersen BN, Soleng A, Okbaldet YB, Pettersson JH, Dudman SG, et al. Prevalence of tick-borne encephalitis virus in *Ixodes ricinus* ticks from three islands in north-western Norway. APMIS. 2015 Sep;123(9):759–64.
363. Zubriková D, Wittmann M, Hönig V, Švec P, Víchová B, Essbauer S, et al. Prevalence of tick-borne encephalitis virus and *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks in Lower Bavaria and Upper Palatinate, Germany. Ticks and Tick-borne Diseases. 2020 May;11(3):101375.
364. Wójcik-Fatla A, Cisak E, Zając V, Zwoliński J, Dutkiewicz J. Prevalence of tick-borne encephalitis virus in *Ixodes ricinus* and *Dermacentor reticulatus* ticks collected from the Lublin region (eastern Poland). Ticks and Tick-borne Diseases. 2011 Mar;2(1):16–9.
365. Belova OA, Burenkova LA, Karganova GG. Different tick-borne encephalitis virus (TBEV) prevalences in unfed versus partially engorged ixodid ticks – Evidence of virus replication and changes in tick behavior. Ticks and Tick-borne Diseases. 2012 Sep; 3(4):240–6.
366. Bormane A, Lucenko I, Duks A, Mavtchoutko V, Ranka R, Salmina K, et al. Vectors of tick-borne diseases and epidemiological situation in Latvia in 1993–2002. International Journal of Medical Microbiology Supplements. 2004 Apr;293:36–47.
367. Korenberg EI, Gorban' LYa, Kovalevskii YV, Frizen VI, Karavanov AS. Risk for Human Tick-Borne Encephalitis, Borrelioses, and Double Infection in the Pre-Ural Region of Russia. Emerg Infect Dis. 2001 Jun;7(3):459–62.
368. Lindblom P, Wilhelmsson P, Fryland L, Sjöwall J, Haglund M, Matussek A, et al. Tick-borne encephalitis virus in ticks detached from humans and follow-up of serological and clinical response. Ticks and Tick-borne Diseases. 2014 Feb;5(1):21–8.
369. Bakhvalova VN, Chicherina GS, Potapova OF, Panov VV, Glupov VV, Potapov MA, et al. Tick-Borne Encephalitis Virus Diversity in Ixodid Ticks and Small Mammals in South-Western Siberia, Russia. Vector-Borne and Zoonotic Diseases. 2016 Aug;16(8): 541–9.
370. Pintér R, Madai M, Horváth G, Németh V, Oldal M, Kemenesi G, et al. Molecular Detection and Phylogenetic Analysis of Tick-Borne Encephalitis Virus in Rodents Captured in the Transdanubian Region of Hungary. Vector-Borne and Zoonotic Diseases. 2014 Aug;14(8):621–4.
371. Achazi K, Růžek D, Donoso-Mantke O, Schlegel M, Ali HS, Wenk M, et al. Rodents as Sentinels for the Prevalence of Tick-Borne Encephalitis Virus. Vector Borne Zoonotic Dis. 2011 Jun;11(6):641–7.
372. Tonteri E, Jääskeläinen AE, Tikkakoski T, Voutilainen L, Niemimaa J, Henttonen H, et al. Tick-borne Encephalitis Virus in Wild Rodents in Winter, Finland, 2008–2009. Emerg Infect Dis. 2011 Jan;17(1):72–5.
373. Brandenburg PJ, Obiegala A, Schmuck HM, Dobler G, Chitimia-Dobler L, Pfeffer M. Seroprevalence of Tick-Borne Encephalitis (TBE) Virus Antibodies in Wild Rodents from Two Natural TBE Foci in Bavaria, Germany. Pathogens. 2023 Jan;12(2):185.
374. Grzybek M, Alsarraf M, Tołkacz K, Behnke-Borowczyk J, Biernat B, Stańczak J, et al. Seroprevalence of TBEV in bank voles from Poland—a long-term approach. Emerging Microbes & Infections. 2018 Dec;7(1):1–8.
375. Kozuch O, Labuda M, Lysý J, Weismann P, Krippel E. Longitudinal study of natural foci of Central European encephalitis virus in West Slovakia. Acta Virol. 1990 Dec;34(6):537–44.
376. Kocianová E, Kozuch O, Bakoss P, Rehácek J, Kovácová E. The prevalence of small terrestrial mammals infected with tick-borne encephalitis virus and leptospirae in the

foothills of the southern Bavarian forest, Germany. Appl Parasitol. 1993 Nov;34(4): 283–90.
377. Zöldi V, Papp T, Reiczigel J, Egyed L. Bank voles show high seropositivity rates in a natural TBEV focus in Hungary. Infectious Diseases. 2015 Mar;47(3):178–81.
378. Bournez L, Umhang GR, Moinet M, Richomme CL, Demerson JM, Caillot C, et al. Tick-borne encephalitis virus in an Alsatian forest, eastern France: seasonal and annual variation of epidemiological parameters related to nymph-to-larva transmission and exposure of small mammals. Pathogens. 2020;9(7):518.
379. Burri C, Korva M, Bastic V, Knap N, Avšič-Županc T, Gern L. Serological Evidence of Tick-Borne Encephalitis Virus Infection in Rodents Captured at Four Sites in Switzerland. J Med Entomol. 2012 Mar;49(2):436–9.
380. Borde JP, Glaser R, Braun K, Riach N, Hologa R, Kaier K, et al. Decoding the Geography of Natural TBEV Microfoci in Germany: A Geostatistical Approach Based on Land-Use Patterns and Climatological Conditions. Int J Envir Res Pub Health. 2022 Sep;19(18):11830.
381. Nosek J, Kožuch O, Grulich I. The structure of tick-borne encephalitis (TBE) foci in Central Europe. Oecologia. 1970 Mar;5(1):61–73.
382. Dobler G, Gniel D, Petermann R, Pfeffer M. Epidemiology and distribution of tick-borne encephalitis. Wien Med Wochenschr. 2012 Jun;162(11):230–8.
383. Zöldi V, Papp T, Rigó K, Farkas J, Egyed L. A 4-Year Study of a Natural Tick-Borne Encephalitis Virus Focus in Hungary, 2010–2013. Ecohealth. 2015 Mar;12(1):174–82.
384. Danielová V, Schwarzová L, Materna J, Daniel M, Metelka L, Holubová J, et al. Tick-borne encephalitis virus expansion to higher altitudes correlated with climate warming. International Journal of Medical Microbiology. 2008 Sep;298:68–72.
385. Bournez L, Umhang G, Moinet M, Boucher JM, Demerson JM, Caillot C, et al. Disappearance of TBEV Circulation among Rodents in a Natural Focus in Alsace, Eastern France. Pathogens. 2020 Nov;9(11):930.
386. Ivaviciute G. The change of natural landscape in Lithuania. In: Research for Rural Development 2020. Vol. 35. [Internet]. [cited 2024 Jan 20]. p. 213–8. Available from: https://llufb.llu.lv/conference/Research-for-Rural-Development/2020/LatviaResRuralDev_26th_2020-213-218.pdf
387. Kikkawa J. Movement, Activity and Distribution of the Small Rodents *Clethrionomys glareolus* and *Apodemus sylvaticus* in Woodland. The Journal of Animal Ecology. 1964 Jun;33(2):259.
388. Kurtenbach K, Kampen H, Dizij A, Arndt S, Seitz HM, Schaible UE, et al. Infestation of Rodents with Larval *Ixodes ricinus* (*Acari; Ixodidae*) Is an Important Factor in the Transmission Cycle of *Borrelia burgdorferi* s.l. in German Woodlands. Journal of Medical Entomology. 1995 Nov;32(6):807–17.
389. Labuda M, Nuttall PA, Kožuch O, Elečková E, Williams T, Žuffová E, et al. Non-viraemic transmission of tick-borne encephalitis virus: a mechanism for arbovirus survival in nature. Experientia. 1993 Sep;49(9):802–5.
390. Fischer C, Schröder B. Predicting spatial and temporal habitat use of rodents in a highly intensive agricultural area. Agriculture, Ecosystems & Environment. 2014 May;189: 145–53.
391. Bolzoni L, Rosà R, Cagnacci F, Rizzoli A. Effect of deer density on tick infestation of rodents and the hazard of tick-borne encephalitis. II: Population and infection models. International Journal for Parasitology. 2012 Apr;42(4):373–81.
392. Jaenson TGT, Petersson EH, Jaenson DGE, Kindberg J, Pettersson JHO, Hjertqvist M, et al. The importance of wildlife in the ecology and epidemiology of the TBE virus in

Sweden: incidence of human TBE correlates with abundance of deer and hares. Parasites Vectors. 2018 Dec;11(1):477.
393. Balčiauskas L, Kawata Y. Red Deer in Lithuania: History, Status and Management. Sustainability. 2022 Oct;14(21):14091.
394. Kučas A, Trakimas G, Balčiauskas L, Vaitkus G. Multi-scale analysis of forest fragmentation in Lithuania. Baltic Forestry. 2011;17(1):128–135.
395. Žygutienė M, Alekseev A, Dubinina H, Kazlauskienė R. Evidence for a risk of tick-borne infection in the city parks of Vilnius, Lithuania. Ekologija. 2008 Jan;54(1):40–3.
396. Leland DS, Ginocchio CC. Role of Cell Culture for Virus Detection in the Age of Technology. Clin Microbiol Rev. 2007 Jan;20(1):49–78.
397. Hematian A, Sadeghifard N, Mohebi R, Taherikalani M, Nasrolahi A, Amraei M, et al. Traditional and Modern Cell Culture in Virus Diagnosis. Osong Public Health and Research Perspectives. 2016 Apr;7(2):77–82.
398. Ponomareva EP, Ternovoi VA, Mikryukova TP, Protopopova EV, Gladysheva AV, Shvalov AN, et al. Adaptation of tick-borne encephalitis virus from human brain to different cell cultures induces multiple genomic substitutions. Arch Virol. 2017 Oct;162(10):3151–6.
399. Růžek D, Vancová M, Tesařová M, Ahantarig A, Kopecký J, Grubhoffer L. Morphological changes in human neural cells following tick-borne encephalitis virus infection. Journal of General Virology. 2009 Jul;90(7):1649–58.
400. Michelitsch A, Fast C, Sick F, Tews BA, Stiasny K, Bestehorn-Willmann M, et al. Long-term presence of tick-borne encephalitis virus in experimentally infected bank voles (*Myodes glareolus*). Ticks and Tick-borne Diseases. 2021 Jul;12(4):101693.
401. Krylova NV, Smolina TP, Leonova GN. Molecular Mechanisms of Interaction Between Human Immune Cells and Far Eastern Tick-Borne Encephalitis Virus Strains. Viral Immunology. 2015 Jun;28(5):272–81.
402. Bakker JW, Pascoe EL, van de Water S, van Keulen L, de Vries A, Woudstra LC, et al. Infection of wild-caught wood mice (*Apodemus sylvaticus*) and yellow-necked mice (*A. flavicollis*) with tick-borne encephalitis virus. Sci Rep. 2023 Dec;13(1):21627.
403. Niedrig M, Aberle SW, Avšič-Županc T, Labuda M, Donoso Mantke O. Quality control assessment for the PCR diagnosis of tick-borne encephalitis virus infections. Journal of Clinical Virology. 2007 Jan;38(1):73–7.
404. Bakhvalova VN, Dobrotvorsky AK, Morozova OV, Matveeva VA, Tkachev SE, et al. Persistence and activation of tick-borne encephalitis virus in small mammals in forest-steep zone of South Siberia. In: Modern situation and perspectives of combat with tick-borne infections in XXI century. Tomsk, Tomsk state university (TSU), 2006. p. 19–21.
405. Bakhvalova VN, Panov VV, Potapova OF, Matveeva LE, Morozova OV. Persistence of tick-borne encephalitis virus in organisms of wild small mammals and in permissive tissue cultures. Far Eastern Journal of Infectious Pathology. 2007;11:79–86.
406. Labuda M, Austyn JM, Zuffova E, Kozuch O, Fuchsberger N, Lysy J, et al. Importance of Localized Skin Infection in Tick-Borne Encephalitis Virus Transmission. Virology. 1996 May;219(2):357–66.
407. Nosek J, Grulich I. The relationship between the tick-borne encephalitis virus and the ticks and mammals of the Tribec mountain range. Bull World Health Organ. 1967;36 Suppl:31–47.
408. Korenberg EI, Kovalevskii YuV. Variation in parameters affecting risk of human disease due to TBE virus. Folia Parasitol (Praha). 1995;42(4):307–12.

409. Hudáčková V, Pekarčíková J, Peťko B, Mikulová K, Sivčo P, Rusňák M. The impact of climatic conditions on the dynamics of tick-borne encephalitis in Slovakia in 2012–2016. Epidemiol Mikrobiol Imunol. 2023;72(2):78–85.
410. Elväng A, Melik W, Bertrand Y, Lönn M, Johansson M. Sequencing of a Tick-Borne Encephalitis Virus from *Ixodes ricinus* Reveals a Thermosensitive RNA Switch Significant for Virus Propagation in Ectothermic Arthropods. Vector-Borne and Zoonotic Diseases. 2011 Jun;11(6):649–58.
411. Moshkin M, Gerlinskaya L, Morozova O, Bakhvalova V, Evsikov V. Behaviour, chemosignals and endocrine functions in male mice infected with tick-borne encephalitis virus. Psychoneuroendocrinology. 2002 Jul;27(5):603–8.
412. Bournez L, Umhang G, Moinet M, Richomme C, Demerson JM, Caillot C, et al. Tick-Borne Encephalitis Virus: Seasonal and Annual Variation of Epidemiological Parameters Related to Nymph-to-Larva Transmission and Exposure of Small Mammals. Pathogens. 2020 Jun;9(7):518.
413. Hughes VL, Randolph SE. Testosterone increases the transmission potential of tick-borne parasites. Parasitology. 2001 Oct;123(4):365–71.
414. Moshkin MP, Novikov EA, Tkachev SE, Vlasov VV. Epidemiology of a tick-borne viral infection: theoretical insights and practical implications for public health. Bioessays. 2009 Jun;31(6):620–8.
415. Frolova TV, Pogodina VV. Activating effect of adrenaline, prednisolone and vincristine in the late periods of tick-borne encephalitis virus persistence. Vopr Virusol. 1984 Feb;29(1):103–8.
416. Romanova LIu, Gmyl AP, Dzhivanian TI, Bakhmutov DV, Lukashev AN, Gmyl LV, et al. Microevolution of tick-borne encephalitis virus in course of host alternation. Virology. 2007 May;362(1):75–84.
417. Asghar N, Lindblom P, Melik W, Lindqvist R, Haglund M, Forsberg P, et al. Tick-Borne Encephalitis Virus Sequenced Directly from Questing and Blood-Feeding Ticks Reveals Quasispecies Variance. PLoS ONE. 2014 Jul;9(7):e103264.
418. Litov AG, Deviatkin AA, Goptar IA, Dedkov VG, Gmyl AP, Markelov ML, et al. Evaluation of the population heterogeneity of TBEV laboratory variants using high-throughput sequencing. Journal of General Virology. 2018 Feb;99(2):240–5.
419. Růžek D, Gritsun TS, Forrester NL, Gould EA, Kopecký J, Golovchenko M, et al. Mutations in the NS2B and NS3 genes affect mouse neuroinvasiveness of a Western European field strain of tick-borne encephalitis virus. Virology. 2008 May;374(2):249–55.
420. Mlera L, Bloom ME. The Role of Mammalian Reservoir Hosts in Tick-Borne Flavivirus Biology. Front Cell Infect Microbiol. 2018 Aug;8:298.
421. Bugarski-Stanojevi V, Blagojevi J, Adnaevi T, Joji V, Vujoevi M. Molecular phylogeny and distribution of three *Apodemus* species (*Muridae, Rodentia*) in Serbia. Journal of Zoological Systematics and Evolutionary Research. 2008 Aug;46(3):278–86.
422. Torre I, Arrizabalaga A. Habitat preferences of the bank vole *Myodes glareolus* in a Mediterranean mountain range. Acta Theriol. 2008 Sep;53(3):241–50.
423. Hubálek Z. Effect of environmental variables on incidence of tick-borne encephalitis, leptospirosis and tularaemia. Cent Eur J Public Health. 2021 Sep;29(3):187–90.
424. Heigl Z, Von Zeipel G. Experimental infection with tick-borne virus in *Clethrionomys glareolus*, *Apodemus flavicollis*, *Apodemus sylvaticus* and *Mus musculus*: 1. Virological Studies. Acta Pathologica Microbiologica Scandinavica. 1966 Jul;66(4):489–509.

425. Tonteri E, Kipar A, Voutilainen L, Vene S, Vaheri A, Vapalahti O, et al. The Three Subtypes of Tick-Borne Encephalitis Virus Induce Encephalitis in a Natural Host, the Bank Vole (*Myodes glareolus*). PLoS ONE. 2013 Dec;8(12):e81214.
426. Stoltz M, Sundström KB, Hidmark Å, Tolf C, Vene S, Ahlm C, et al. A Model System for In Vitro Studies of Bank Vole Borne Viruses. PLoS ONE. 2011 Dec;6(12):e28992.
427. Frey S, Essbauer S, Zöller G, Klempa B, Dobler G, Pfeffer M. Full genome sequences and preliminary molecular characterization of three tick-borne encephalitis virus strains isolated from ticks and a bank vole in Slovak Republic. Virus Genes. 2014 Feb;48(1):184–8.
428. Weidmann M, Schmidt P, Hufert FT, Krivanec K, Meyer H. Tick-Borne Encephalitis Virus in *Clethrionomys glareolus* in the Czech Republic. Vector-Borne and Zoonotic Diseases. 2006 Dec;6(4):379–81.
429. Zeipel GV, Heigl Z. Experimental infection with tick-borne virus in *Clethrionomys glareolus*, *Apodemus flavicollis*, *Apodemus sylvaticus* and *Mus musculus*: 2. Serological Studies. Acta Pathologica Microbiologica Scandinavica. 1966 Nov;68(2):211–29.
430. Yun SM, Kim SY, Ju YR, Han MG, Jeong YE, Ryou J. First complete genomic characterization of two tick-borne encephalitis virus isolates obtained from wild rodents in South Korea. Virus Genes. 2011 Jun;42(3):307–16.
431. Egyed L, Zöldi V, Szeredi L. Subclinical Tick-Borne Encephalitis Virus in Experimentally Infected *Apodemus agrarius*. Intervirology. 2015;58(6):369–72.
432. Kopecký J, Tomková E, Vlcek M. Immune response of the long-tailed field mouse (*Apodemus sylvaticus*) to tick-borne encephalitis virus infection. Folia Parasitol (Praha). 1991;38(3):275–82.
433. Yoshii K, Mottate K, Omori-Urabe Y, Chiba Y, Seto T, Sanada T, et al. Epizootiological Study of Tick-Borne Encephalitis Virus Infection in Japan. J Vet Med Sci. 2011;73(3):409–12.
434. Bakhvalova VN, Potapova OF, Panov VV, Morozova OV. Vertical transmission of tick-borne encephalitis virus between generations of adapted reservoir small rodents. Virus Research. 2009 Mar;140(1–2):172–8.
435. Molnárová A, Mayer V. Experimental infection of pregnant mice with viruses of the tick-borne encephalitis (TBE) complex. Acta Virol. 1980 Jun;24(4):297.
436. Vaisvieliene D. TBE in Lithuania. In: Süss, J, Kahl O, editors. Proceedings of the Fourth International Potsdam Symposium on Tick-Borne Diseases; Potsdam, Germany; February 21–22, 1997. Lengerich (Germany): Pabst Science Publishers; 1997. p. 100–13.
437. Charlier C, Beaudoin MC, Couderc T, Lortholary O, Lecuit M. Arboviruses and pregnancy: maternal, fetal, and neonatal effects. The Lancet Child & Adolescent Health. 2017 Oct;1(2):134–46.
438. Gerlinskaia LA, Bakhvalova VN, Morozova OV, Tsekhanovskaia NA, Matveeva VA, Moshkin MP. Sexual transmission of tick-borne encephalitis virus in laboratory mice. Biull Eksp Biol Med. 1997 Mar;123(3):327–8.

LIST OF PUBLICATIONS

1. **Šimkutė**, **Evelina**; Pautienius, Arnoldas; Grigas, Juozas; Sidorenko, Marina; Radzijevskaja, Jana; Paulauskas, Algimantas; Stankevičius, Arūnas. The Prevalence of Tick-Borne Encephalitis Virus in Wild Rodents Captured in Tick-Borne Encephalitis Foci in Highly Endemic Lithuania // Viruses, 2024, t. 16, nr. 3, p. 1–14, ISSN 1999-4915. doi: 10.3390/v16030444. Prieiga per internetą: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10974453/ https://hdl.handle.net/20.500.12512/242473 <10.3390/v16030444>. Science Citation Index Expanded (Web of Science); Scopus; PubMed; PubMed Central; MEDLINE. [S1] [M.kr.: A002] [Citav. rodiklis: 4.7, bendr. cit. rod.: 5.9, kvartilis: Q2 (2022. InCites JCR SCIE)]
2. **Šimkutė**, **Evelina**; Pautienius, Arnoldas; Grigas, Juozas; Urbutė, Paulina; Stankevičius, Arūnas. The Prevalence, Seroprevalence, and Risk Factors of Tick-Borne Encephalitis Virus in Dogs in Lithuania, a Highly Endemic State // Viruses, 2023, t. 15, nr. 11, p. 1-12, ISSN 1999-4915. doi:10.3390/v15112265. Prieiga per internetą: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10674385/ https://hdl.handle.net/20.500.12512/239708 <10.3390/v15112265>. Science Citation Index Expanded (Web of Science); PubMed; PubMed Central; Scopus. [S1] [M.kr.: A002] [Citav. rodiklis: 4.7, bendr. cit. rod.: 5.9, kvartilis: Q2 (2022. InCites JCR SCIE)]

List of scientific conference abstracts

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COPIES OF PUBLICATIONS

Article

The Prevalence, Seroprevalence, and Risk Factors of Tick-Borne Encephalitis Virus in Dogs in Lithuania, a Highly Endemic State

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Abstract: The rising awareness and increasing number of case reports of tick-borne encephalitis (TBE) in dogs indicate that the virus might be an important tick-borne pathogen in dogs, especially in endemic areas. Therefore, the aim of the present study was to investigate the prevalence rate of TBEV RNA and TBEV-specific antibodies in clinical samples of dogs living in a highly endemic region of Lithuania and to evaluate the main risk factors for severe disease course and death. The blood samples (n = 473) of dogs were collected in two veterinary clinics in central Lithuania. Tick-borne encephalitis virus (TBEV) RNA was detected in 18.6% (88/473; CI 95% 15.2–22.4) and TBEV-specific antibodies were found in 21.6% (102/473; CI 95% 17.9–25.6) of dog blood serum samples after confirmation with a virus neutralization test. The death/euthanasia rate was 18.2% (16/88; CI 95% 10.8–27.8) in PCR-positive dogs. Male dogs were more likely to develop neurological symptoms (p = 0.008). Older dogs (p = 0.003), dogs with the presence of neurological symptoms (p = 0.003), and dogs with the presence of TBEV-specific antibodies (p = 0.024) were more likely to experience worse outcomes of the disease. The results of the present study demonstrate that TBEV is a common and clinically important pathogen in dogs in such endemic countries as Lithuania.

Keywords: tick-borne encephalitis; TBEV prevalence; TBEV seroprevalence; TBEV in dogs; RT-nPCR

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1. Introduction

Tick-borne encephalitis virus (TBEV) is one of the most important tick-borne viruses in human medicine. The virus can cause neurological symptoms with long-term health impairment and can be fatal [1]. TBEV belongs to the family *Flaviviridae* and is endemic in Europe and Northeastern Asia [2]. For the past decade, Lithuania has recorded one of the highest prevalence rates of human TBE cases in Europe [3]. Although many species of animals can be infected by TBEV, clinical symptoms are mainly observed in humans and less commonly in horses and dogs [4].

In endemic areas, TBE is considered one of the most important infections of the central nervous system (CNS) in dogs [5]. In some endemic locations of Europe, the seroprevalence of TBEV-specific antibodies in dogs has been reported to be 30–40% [6–8]. Studies suggest that the majority of TBE cases in dogs are asymptomatic, although cases of peracute, acute, and chronic courses of the disease were reported [9–11]. The clinical symptoms of TBE in dogs are similar to the symptoms reported in human patients and a biphasic course of the disease is prominent in dogs [5,12]. At the first stage, fever, apathy, anorexia, and gastrointestinal symptoms are mainly reported both in human patients and in dogs. If the second stage of TBE develops, neurological symptoms are mainly observed [5,13]. Although neurological symptoms in dogs living in TBEV-endemic regions strongly imply TBE as a differential diagnosis, studies show that TBE is underdiagnosed in dogs [11,14,15].

Currently, the diagnosis of TBE mainly relies on the detection of specific IgM antibodies in cerebrospinal fluid (CSF) and/or in blood serum samples [3]. Although cross-reactions

with other flaviviruses are conceivable, an increase in IgG antibodies evaluated with an ELISA in recollected samples might aid in the diagnosis of TBE [16]. For an early differential diagnosis of TBE, viral RNA detection using PCR might be a valuable tool [3]. In addition, the presence of neurological clinical symptoms, anamnesis of tick-bite, and living or visiting TBE endemic areas may all point to TBE [17].

The present study sought to investigate the prevalence of TBEV RNA and TBEV-specific antibodies in dogs living in Lithuania for the first time, as well as to evaluate the risk factors for severe disease course and mortality.

2. Materials and Methods

2.1. Sample Collection and Data of Anamnesis

Two veterinary clinics located in Kaunas (central part of Lithuania) agreed to participate in the present study. Prior to conducting the investigation, explicit consent was obtained from the owners of the dogs, granting authorization for the utilization of clinical samples. Randomly collected blood samples from dogs (n = 473) with various diseases were obtained from clinics between May 2020 and December 2021. Additionally, 3 samples of cerebrospinal fluid (CSF) were collected and analyzed. All samples were stored at −80 °C for further analysis. Data on clinical anamnesis and outcome of disease were collected retrospectively starting one month before sample collection and followed up to six months thereafter. Dogs of 45 different breeds were included in the study and the most common anamnesis was apathy, inappetence, and gastrointestinal symptoms (Supplementary Table S1). Concurrent tick-borne diseases were diagnosed at veterinary clinics using blood smears, focusing on the detection of *Babesia* spp. and *Anaplasma phagocytophilum*. Anti-*A. phagocytophilum* antibodies were detected by chromatographic immunoassay test (Anigen Rapid CaniV-4 Test Kit (BioNote, INC. Hwaseong, Republic of Korea). The attached ticks (n = 117) were collected when they were found on the bodies of dogs at the same visit at the veterinary clinic when blood serum samples were collected. Each tick was individually treated with liquid nitrogen, homogenized using mortar and pestle, mixed with 1000 μL of Modified Eagle's Medium (MEM, Gibco, Waltham, MA, USA), and stored at −80 °C for further analysis.

2.2. Sample Analysis with ELISA, VNT, and RT-nPCR

The serum samples were analyzed using commercially available quantitative enzyme-linked immunosorbent assay (ELISA) (VetLine TBE/FSME ELISA, NovaTec Immundiagnostica GmbH, Dietzenbach, Germany) according to the manufacturer's instructions. To confirm positive ELISA results, a virus neutralization test was performed according to the instructions described before [18].

Total RNA was extracted from blood serum (n = 473) and CSF (n = 3) samples using GeneJET RNA Purification Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Serum and whole blood samples of dogs and suspensions of ticks and isolates in cell cultures were analyzed with reverse transcription nested polymerase chain reaction assay (RT-nPCR) using DreamTaq Green PCR Master Mix and primers as previously described [19].

2.3. Virus Isolation

The suspensions of ticks, blood serum, and CSF samples were centrifuged at 12.000 g for 5 min and filtered with a 0.22 μm pore size microfilter (Techno Plastic Products AG, Trasadingen, Switzerland). Cell cultures of Vero (ATCC No. CCL-81) and Marc-145 (ATCC No. CRL-12231) were seeded in a maintenance medium containing Modified Eagle's Medium (MEM, Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) was used for murine neuroblastoma cells (Neuro-2a ATCC No. CCL-131) instead of MEM. All cells were incubated at 37 °C in 5% CO_2 overnight. Cells were inoculated with prepared tick suspensions or clinical samples

from the dogs the following day. Cell cultures were examined for the occurrence of cytopathic effects through three serial passages which were performed in triplicates including triplicates of positive and negative controls for each round of analysis. After each serial passage, cell suspensions were harvested for RNA extraction. Successful virus isolation in cell cultures was confirmed using RT-nPCR. Partial genome sequencing targeting the NCR region of TBEV was used for internal confirmation of the specificity of detected TBEV RNA.

2.4. Statistical Analysis

The confidence intervals were calculated using the exact binomial method. To determine the significance of the risk variables, binary logistic regression analysis and the chi-square test were used. Standardized residuals were employed as post-hoc analysis after statistically significant chi-square tests. The statistical analysis and visualizations were performed utilizing the programming language R 4.3.2.

3. Results

3.1. Prevalence of TBEV and TBEV-Specific Antibodies in Dogs

A total number of 473 dog blood samples were analyzed using RT-nPCR and ELISA in the present study. Viral RNA was detected in 18.6% (88/473; CI 95% 15.2–22.4) of dog blood/serum samples. In addition, viral RNA was detected in two out of three CSF samples. TBEV-specific antibodies were detected in 21.6% (102/473; CI 95% 17.9–25.6) of dog blood serum samples after confirmation with a virus neutralization test (Figure 1A). No statistically significant associations were found between the antibody and TBEV RNA prevalence rates in dog blood serum samples based on sample collection time (Figure 1B).

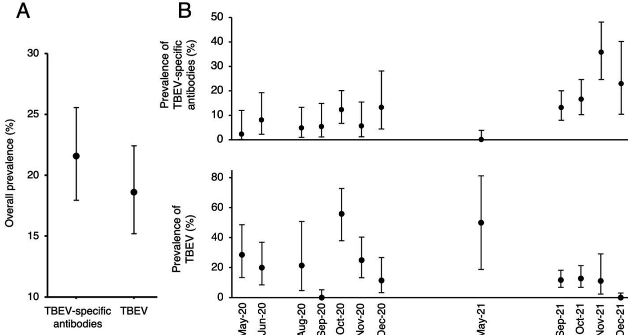

Figure 1. (**A**) The prevalence rate (%) of TBEV RNA and TBEV-specific antibodies in blood/blood serum samples of dogs and (**B**) prevalence rate (%) in different months of sample collection.

The median age of PCR-positive dogs was 7 years, ranging from 2 months to 15 years. The rate of death/euthanasia was 18.2% (16/88; CI 95% 10.8–27.8) in PCR-positive dogs. Because of severe neurological symptoms or poor prognosis, 12.5% (11/88; CI 95% 6.4–21.3) of PCR-positive dogs were euthanized while the remaining 5.7% (5/88; CI 95% 1.9–12.8) died regardless of intense care and symptomatic treatment. Tick bites were reported by owners in 29.6% (26/88; CI 95% 20.3–40.2) of PCR-positive dogs over a two-week period. Other tick-borne diseases were concurrently diagnosed in 19.3% (17/88; CI 95% 11.7–29.1) of PCR-positive dogs (*Babesia* spp. and *A. phagocytophilum* were diagnosed in 17 and 3 dogs, respectively). In addition, dogs of 36 different breeds were found to be PCR-positive, including small dog breeds.

TBEV-specific antibodies were detected in 26.1% (23/88; CI 95% 17.3–36.6) of PCR-positive dogs with a mean antibody level of 189 NTU/mL (range 29.7–525 NTU/mL). The

median age of seropositive viremic dogs was 8 years, ranging from 1 to 14 years. Dogs older than 1 year of age were found to be seropositive and viremic statistically significantly more often than dogs under the age of 1 year (37.5% (95% CI 26.3–49.7) and 0%, respectively, (p = 0.003)). In addition, 16.7% (79/473; CI 95% 13.5–20.4) of the dogs in the study were seropositive but PCR-negative.

3.2. Risk Factors for Clinical TBE Infection in Dogs and Lethal Outcomes

Statistical analysis revealed that male dogs (43.7% (95% CI 29.5–58.8)) were more likely (p = 0.008) to develop neurological symptoms compared to females (17.5% (95% CI 7.3–33.0)). The lethal outcome/euthanasia of PCR-positive dogs with neurological symptoms was associated with older age. The mean age of recovered and deceased/euthanized dogs was 5.6 and 11.8 years, respectively (p = 0.003). Other causes of death/euthanasia, such as neoplastic processes, trauma, or hydrothorax were not related to the age of PCR-positive dogs. Moreover, dogs with neurological symptoms had a higher risk of experiencing a worse outcome (χ^2; = 11.57, df = 2, p = 0.003) resulting in a significantly lower number of recovered dogs with neurological symptoms (27.8% (95% CI 19.3–36.6)) compared to the number of recovered dogs without neurological symptoms (72.2% (95% CI 63.7–80.7)).

The chi-square test of independence revealed a statistically significant association between the antibody status and the outcome of the disease (χ^2; = 5.05, df = 1, p = 0.024). Subsequent post-hoc analysis revealed that the frequencies of recovered dogs were lower by 14.2% in the seropositive group and higher by 8.6% in the seronegative group as compared to the predicted model findings. In contrast, the incidences of death/euthanasia were 65.5% higher in seropositive dogs and 39.7% lower in seronegative dogs. The results imply that the presence of TBEV-specific antibodies in the blood serum of PCR-positive dogs might be related to a higher risk of death/euthanasia.

3.3. TBEV Seroprevalence and Associations with Clinical Symptoms and the Presence of other Tick-Borne Diseases

Out of the 102 seropositive dog blood serum samples confirmed with a virus neutralization test, 13.7% (14/102; CI 95% 7.7–22) were considered borderline (20–30 NTU/mL) with an ELISA test, and 86.3% (88/102; CI 95% 78–92.3) were positive (>30 NTU/mL) (Figure 2A–C). Statistical analysis revealed significantly higher antibody levels in dogs with potentially TBE-related symptoms compared to dogs with non-specific symptoms (p = 0.002) and dogs with non-TBE-related symptoms (p = 0.001) (Figure 2D).

Other tick-borne diseases were diagnosed in 14.7% (15/102; CI 95% 8.5–23.1) of dogs with TBE-specific antibodies: protozoa of *Babesia* spp. were found in blood samples of 13 dogs and 2 dogs had specific antibodies against *A. phagocytophilum*. Moreover, dogs diagnosed with other tick-borne diseases had significantly (p = 0.046) higher levels of TBEV-specific antibodies (Figure 2E).

Clinical neurological symptoms potentially related to TBE were found in 13.7% (14/102; CI 95% 7.7–22) of seropositive dogs. Non-specific symptoms such as hyperthermia, apathy, and gastrointestinal symptoms (vomiting and/or diarrhea) were observed in 32.4% (33/102; CI 95% 23.4–42.3) of seropositive dogs. Other diseases were diagnosed in 39.2% (40/102; CI 95% 29.7–49.4) of seropositive dogs with various symptoms that were not considered to be associated with TBE (skin allergies, pyometra, kidney or liver failure, trauma, oncologic processes, and other diseases).

On physical examination, neurological symptoms were observed in 31.8% (28/88; CI 95% 22.3–42.6) of PCR-positive dogs. TBEV-specific antibodies were detected in 32.1% (9/28; CI 95% 15.9–52.4) of PCR-positive dogs with neurological symptoms. The dogs with neurological symptoms had a median age of 8 years, ranging from 4 months to 15 years. Neurological symptoms were considered moderate or severe in 12.5% (11/88; CI 95% 6.4–21.3) of viremic dogs. Episodes of seizures, tetraparesis, impaired vision, deficits of facial reflexes, altered mental status, behavior changes, and cognitive function impairment were observed. Five of six dogs with seizures died, four of which did not show

hyperthermia, thus implying that the absence of fever in the presence of severe neurological symptoms might be related to a worse outcome of TBE. Mild neurological symptoms were present in 19.3% (17/88; CI 95% 11.7–29.1) of PCR-positive dogs. Difficulties standing up, reluctance to walk, presence of a stiff walk, back hyperesthesia (without organic defects in the musculoskeletal system), and impaired coordination were observed.

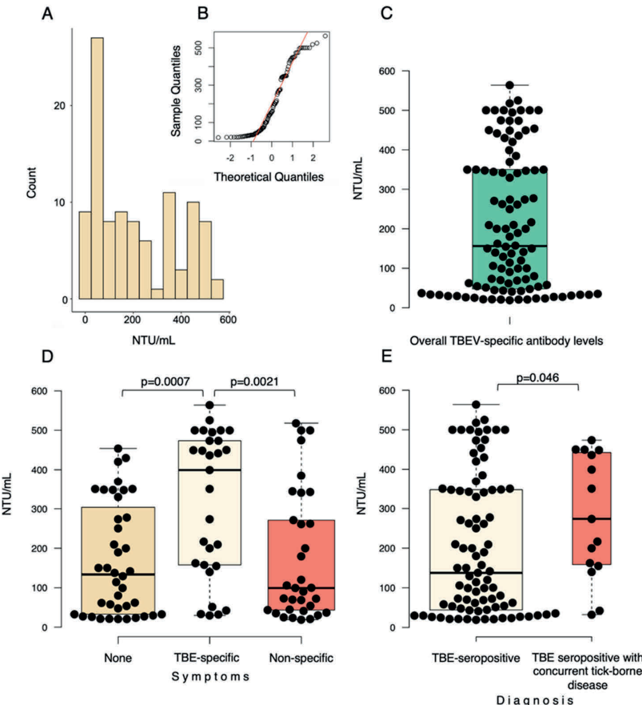

Figure 2. Prevalence of TBEV−specific antibodies. (**A**) Number of seropositive dogs and levels of detected TBEV−specific antibodies. (**B**) Q−Q plot. (**C**) Differences in levels of TBEV−specific antibodies in seropositive dogs. (**D**) Differences in levels of TBEV−specific antibodies in dogs with potentially TBE−specific symptoms, non−specific symptoms, and dogs with non−TBE−related symptoms. (**E**) Differences in levels of TBEV−specific antibodies in dogs with other tick−borne diseases and dogs without other concurrently diagnosed tick−borne diseases.

In 68.2% (60/88; CI 95% 57.4–77.7) of PCR-positive dogs, no TBE-related neurological symptoms were noticed. Various concurrent diseases were diagnosed in 25% (22/88; CI 95% 16.4–35.4) of dogs. Gastrointestinal symptoms (vomiting/diarrhea) were present in 37.5% (33/88; CI 95% 27.4–48.5) of PCR-positive dogs (of them, 17.1% (15/88; CI 95% 9.9–26.6) of dogs also had neurological symptoms). Other tick-borne diseases were diagnosed in 13.6% (12/88; CI 95% 7.3–22.6) of dogs. Non-specific symptoms such as apathy and loss of appetite were observed in 11.4% (10/88; CI 95% 5.6–19.9) of PCR-positive dogs. Hyperthermia was present in 37.5% (33/88; CI 95% 27.4–48.5) of PCR-positive dogs: 15.9% (14/88; CI 95% 9–25.3) were diagnosed with other concurrent tick-borne diseases; 13.6% (12/88; CI 95% 7.3–22.6) had TBE-related symptoms; and the remaining 10.2% (9/88; CI

95% 4.8–18.5) of dogs had other possible reasons for fever (trauma, neoplastic processes, and other).

Neurological symptoms were present in 12.7% (10/79; CI 95% 6.2–22.1) of seropositive and PCR-negative dogs. Behavior changes were present in two dogs: One dog was found by its owners in a hypersensitive state and was hyperactive, disoriented, and injured himself. The episodes of hypersensitivity lasted for a few days and an episode of impaired vision was present. Another dog was hyperactive and anxious and had episodes of extremely increased appetite for eating grass and licking carpets. Both dogs had high antibody levels (473 NTU/mL and 494 NTU/mL, respectively) and both dogs recovered. Mild neurological symptoms were present in 8.9% (7/79; CI 95% 3.6–17.4) of seropositive dogs and one dog showed uncoordinated movements and episodes of falling down.

No potentially TBE-related symptoms were noticed in 48.1% (38/79; CI 95% 36.7–59.6) of seropositive and PCR-negative dogs. Non-specific symptoms (apathy and loss of appetite) were present in 29.1% (23/79; CI 95% 19.4–40.4) of dogs without final diagnosis and gastrointestinal symptoms were present in 26.6% (21/79; CI 95% 17.3–37.7) of seropositive and PCR-negative dogs.

3.4. TBEV RNA Prevalence in Ticks, Collected from Dogs

Attached *Ixodes ricinus* (n = 112) and *Dermacentor reticulatus* (n = 5) ticks were collected from the dogs and tick suspensions were analyzed using RT-nPCR. Concurrently, tick suspensions were inoculated on Neuro-2a, Vero, and Marc-145 cells. Overall, TBEV RNA was detected in 34.2% (40/117; CI 95% 25.7–43.5) of tick suspension samples (33.9% (38/112; CI 95% 25.8–43.1) in *Ixodes ricinus* and 40.0% (2/5; CI 95% 11.7–76.9) in *Dermacentor reticulatus*). The number of positive samples increased significantly (p = 0.001) to 56.4% (66/117; CI 95% 46.9–65.6) in Marc-145, 58.1% (68/117; CI 95% 48.6–67.2) in Vero, and 60.7% (71/117; CI 95% 51.2–69.6) in Neuro-2a cells after three serial passages (Figure 3A,B). However, no statistically significant relations were found between the viral RNA present in the dog blood samples and the ticks, collected from the respective dogs.

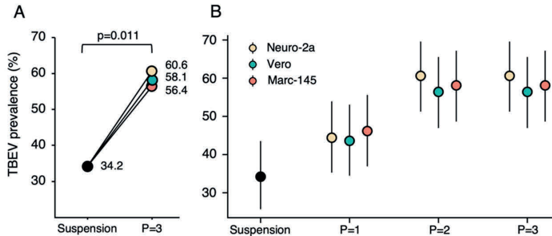

Figure 3. (**A**) Comparison of the prevalence rates (%) of TBEV RNA in tick suspensions and third passage isolates in Neuro-2a, Vero, and Marc-145 cell cultures. (**B**) TBEV RNA prevalence rate (%) changes at each passage in different cell cultures.

3.5. Virus Isolation from PCR-Positive Dogs' Serum and CSF Samples

Due to a short viremia and rapid viral clearance, successful attempts to isolate TBEV in cell cultures from clinical dog samples are rare. However, we successfully isolated TBEV from the blood serum (n = 7) and CSF (n = 1) samples of PCR-positive dogs in Neuro-2a, Vero, and Marc-145 cell cultures. Successful virus isolation was confirmed using RT-nPCR after the second or third passage in the cells.

4. Discussion

The diagnosis of TBE currently relies mainly on detecting TBEV-specific antibodies in CSF or in blood serum samples [3]. However, serological tests may be negative in the early phases of TBE, as was demonstrated in the present study with only 26.1% (23/88; CI 95%

17.3–36.6) of PCR-positive dogs being seropositive for TBEV. In addition, cross-reactions with IgG antibodies of other flaviviruses are possible [16]. The detection of TBEV RNA in 18.6% (88/473) of the randomly collected dog blood samples is in accordance with the results of other authors. A similar study conducted in the Czech Republic reported a prevalence rate of 12.6% (20/159) of PCR-positive dogs in the TBE-endemic region [20]. The high TBEV RNA prevalence in dogs might be related to reduced or delayed immune response due to various diseases such as babesiosis, neoplasia, or chronic diseases. Also, treatment with corticosteroids might be related to longer-lasting viremia [21]. In addition, the age, breed, physiological and immune status of the dog, the specific TBEV strain, or infectious dose may also play an important role in predisposing clinical TBE [22]. Although TBEV RNA detection using PCR is not the preferred method to confirm TBE infection in humans or dogs, Saksida et al. [23] concluded that RT-PCR is an efficient method for an early diagnosis of TBE while specific antibodies are still undetectable. Therefore, the findings suggest that viral RNA detection using PCR might be a valuable tool in diagnosing TBE in dogs in endemic regions, especially in the early or acute stages of the disease.

TBEV-specific antibodies were detected in 21.6% (102/473) of the randomly collected dog blood samples and in 32.1% (9/28) of PCR-positive dogs with neurological symptoms. Although the results are consistent with reports from other European countries, a substantially greater seroprevalence of 37.5% (113/301) was found in horses in a prior investigation in Lithuania [24]. In healthy dogs, the highest reported prevalence rate of TBE-specific antibodies detected using ELISA was 30.4% (17/54) in endemic regions of Germany [8], 30.4% (38/125) in Denmark [7], and 40% (8/20) in the Åland Islands of Finland [6]. However, VNT confirmed the ELISA results only in the Danish island investigation. Following the viral neutralization test, the seroprevalence rate was 4.8% (6/125) in only one area in Bornholm, a Danish island in the Baltic Sea [7], implying that the true seroprevalence might be lower in other countries as well. The reported prevalence rate of TBEV-specific antibodies in dogs with neurological symptoms varied between 11.3% (18/159) in the blood serum samples of dogs from an endemic region in the Czech Republic [20]; 20.2% (110/545) in Austria [25], and 53.6% (30/56) in Germany (30.4% (17/56) also had detectable antibodies in CSF [8]. The results of high seroprevalence show that TBEV in dogs in Lithuania is a common tick-borne pathogen, similar to other TBEV-endemic areas.

A death/euthanasia rate of 18.2% (16/88; CI 95% 10.8-27.8) is much lower than the 33.3% (18/54) reported by Kleeb et al. [5]. We, on the other hand, analyzed randomly collected samples of dogs, while Kleeb et al. [5] conducted a retrospective study of confirmed TBE cases in dogs with clinical symptoms. In the present study, all PCR-positive dogs under the age of 1 year were TBEV seronegative. The most likely explanation is that these dogs were infected with TBEV for the first time in their life and antibodies were not detectable at the time of testing. A study conducted by Salat et al. [26] found a statistically significant correlation between age and VNT seropositivity. However, the data on TBEV seroprevalence in dogs under the age of 1 year is scarce because older dogs are mainly included in the studies since they are more likely to have had previous contact with ticks and, therefore, tick-borne pathogens [7,26]. In addition, dogs of small breeds (less than 15 kg weight) were omitted from some of the TBE seroprevalence studies for the same reason as young dogs [7]. However, the results of our study show that differential diagnosis of TBE in endemic areas should not be ruled out due to the young age or small size of the dog.

We looked at the putative risk factors of PCR-positive dogs that might be associated with the severity of clinical neurological symptoms and worse outcomes of the disease. Statistical analysis revealed that male dogs were prone to developing neurological symptoms. Older dogs and dogs with neurological symptoms were more likely to experience worse outcomes, compared to younger dogs and dogs without neurological symptoms. Furthermore, PCR-positive and seropositive dogs (mainly with severe clinical symptoms) were more likely to experience worse outcomes, possibly because of a delayed antibody response in the acute monophasic course of infection when neurological symptoms develop

in the presence of viremia. In accordance with our findings, Kleeb et al. [5] reported that older dogs and dogs with seizures had an increased risk of death. Studies in human patients concluded that older age and male gender were associated with a more severe course of the disease [27,28]. In addition, the delayed immune response of TBEV-Ig antibodies and the monophasic course of the disease were the predictor factors of the severe form of TBE infection in human patients [29,30].

Significantly higher antibody levels were detected in the serum samples of dogs with possibly TBE-related symptoms compared with dogs without TBE-related symptoms. Most likely, these dogs could have had a recent TBE infection which was asymptomatic or not suspected and diagnosed. TBE is thought to be underdiagnosed in dogs, owing to mild or non-specific clinical symptoms or unawareness of the possible disease in severe cases [9,11]. Coinfection with other tick-borne pathogens was diagnosed in 19.3% (17/88; CI 95% 11.7–29.1) of TBEV PCR-positive dogs. In addition, dogs diagnosed with other concurrent tick-borne diseases were more likely to have TBEV-specific antibodies and the levels of antibodies were higher. These findings suggest that dogs with a clinical history of several tick-borne diseases are possibly living in or visiting areas with a high risk of tick-bite and tick-borne infections and therefore could be used as indicators to evaluate the risk of tick-borne zoonoses in humans. It is known that dogs are 50–100 times more likely than human beings to come into contact with TBEV-infected ticks [9]. Furthermore, dogs are known sentinels for TBE foci detection and were used as TBE indicators in countries previously considered to be TBE-free [31].

To the best of our knowledge, we are the first to report cases of concurrent TBE and active *Anaplasma phagocytophilum* infections diagnosed in dogs. Three dogs (3.4% (3/88); CI 95% 0.7–9.6)) were found to be TBEV PCR-positive and concurrently diagnosed with *A. phagocytophilum* infection. However, there are previously reported clinical TBE cases in dogs with high antibody levels against *A. phagocytophilum* and *Ehrlichia canis* in blood serum [32,33]. Concurrent TBE and *Babesia* spp. infections in dogs were reported previously in a case report [15]. Nonetheless, the results of our study indicate that coinfection of tick-borne pathogens in dogs might be underdiagnosed, especially in countries endemic to several tick-borne pathogens.

Isolation of TBEV from tick suspensions in cell cultures significantly increased the number of PCR-positive samples after two serial passages. The results indicate that viral loads in ticks might be too low to be successfully detected using PCR. The prevalence of TBEV RNA in questing ticks is generally less than 1%, although, in some regions in Europe, it has been found to be 2.6–10.8% [34–36]. While Belova's et al. [37] experiment suggests that viral load increases when ticks are feeding on blood, our study found no statistically significant associations between TBE virus presence in dogs and ticks, collected from respective dogs. One possible explanation is that the dogs were infected by ticks that were not collected at the time of the study. Moreover, the period of time of the blood meal or the viral load in the ticks could have been insufficient to infect some of the dogs [38]. A study in neighboring Latvia revealed a high TBEV prevalence in questing *I. ricinus* ticks which varied between 1.7% and 26.6% in 2001 and 1995, respectively, and the prevalence in ticks removed from humans was even higher, reaching 13.2% and 40.9% in 1997 and 1999, respectively [39]. In contrast, a study conducted by Korenberg et al. [40] revealed a higher TBEV prevalence in questing ticks (10.9–38.7%) compared to ticks removed from humans (5.5–11.0%). However, they used different methods for the detection of TBEV: In questing ticks, TBEV was detected after isolation in PK cells and confirmed by direct immunofluorescence test (and additional tests), and TBEV in ticks removed from humans was detected by indirect immunofluorescence assay [40]. Our study shows that virus isolation in cell culture is a more effective method to increase viral load in ticks to detectable levels compared to virus replication in the ticks' salivary glands during a blood meal. The main reason might be an insufficient length of time for the tick blood meal because most people remove ticks promptly (in around 90% of cases), and reactivation, which is required for some microorganisms before infectivity is attained, does not have a sufficient amount of

time to develop [40]. Moreover, other factors, such as host immune response, might have an influence. A study conducted in Sweden analyzed the prevalence of TBEV in detached ticks from human patients and the clinical response of the tick-bitten participants. Two persons developed antibodies against TBEV and one of the patients reported clinical symptoms, despite a lack of TBEV in detached ticks. In addition, one patient did not experience clinical symptoms and did not have antibodies against TBEV, despite the detached tick containing TBEV (1800 copies) [38].

In the present study, the clinical symptoms of PCR-positive dogs were similar to those documented in case reports and other studies of TBE in dogs. The most common symptoms of the first phase of TBE are fever, apathy, and anorexia. Neurological symptoms such as seizures, altered behavior, and consciousness, ataxia/vestibular symptoms, plegia/paresis of one or more limbs, reduced limb muscle tone and spinal reflexes, neck hyperalgesia, cranial nerve deficits, nystagmus, optic neuritis, inability to swallow, and other symptoms were reported in dogs with TBE infection [5,10,32]. Although optic neuritis is a rarely occurring clinical symptom of TBE in dogs, two cases have been previously described [15,32]. In the present study, four dogs could be suspected to have optic neuritis. Three PCR-positive dogs and one dog with high levels of TBE-specific antibodies had transient vision impairment which improved after treatment with corticosteroids. Thus, in endemic areas, TBEV should be considered a possible cause of vision impairment in dogs. Neurological symptoms were present in 31.8% (28/88; CI 95% 22.3–42.6) of PCR-positive dogs. In accordance with our findings, a study conducted in the endemic region of the Czech Republic found that 35% (7/20) of PCR-positive dogs had neurological clinical symptoms [20]. Gastrointestinal symptoms such as vomiting and diarrhea were observed in 37.5% (33/88; CI 95% 27.4–48.5) of PCR-positive dogs. Similar prevalence rates of gastrointestinal symptoms were reported in human TBE patients. A study conducted by Zambito Marsala et al. [41] reported vomiting in 30% (27/89) of cases and nausea in 24.7% (22/89) of cases in human patients. Mickienė et al. [42] reported gastrointestinal complaints in 21.3% (20/94) of TBE patients. The absence of hyperthermia in dogs with seizures was related to a worse outcome. Similarly, a retrospective study of TBE in dogs concluded that hyperthermia was related to a better outcome of disease [5].

5. Conclusions

The revealed results of the presence of neurological symptoms in 31.8% (28/88; CI 95% 22.3–42.6) and a death rate of 18.2% (16/88; CI 95% 10.8-27.8) of PCR-positive dogs implies that TBE is a clinically important disease in dogs in such endemic countries as Lithuania. However, TBE is underdiagnosed in dogs because of a lack of awareness about the disease even in TBE-endemic areas. The present study shows that symptomatic treatment and intensive care might be necessary in severe cases and the treatment is more effective in young dogs without concurrent diseases. The timely diagnosis of TBE might help to apply more effective treatment and supportive care strategies and save at least some of the dogs from euthanasia. TBE is therefore recommended to be included in the differential diagnosis of CNS diseases in dogs in endemic areas.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v15112265/s1, Table S1: Data of Anamnesis of PCR-Positive Dogs.

Author Contributions: Conceptualization, E.S. and A.S.; methodology, E.S., A.P. and A.S.; software, A.P.; validation, E.S., A.P. and A.S.; formal analysis, A.P.; investigation, E.S., A.P., J.G., P.U. and A.S.; resources, A.S.; data curation, E.S. and A.P.; writing—original draft preparation, E.S.; writing—review and editing, A.P., J.G. and A.S.; visualization, A.P.; supervision, A.S.; project administration, A.S.; funding acquisition, E.S. and A.S. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Explicit consent was obtained from the owners of the dogs, granting authorization for the utilization of clinical samples.

Data Availability Statement: Data will be made available on request.

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References

1. Ruzek, D.; Avšič Županc, T.; Borde, J.; Chrdle, A.; Eyer, L.; Karganova, G.; Kholodilov, I.; Knap, N.; Kozlovskaya, L.; Matveev, A.; et al. Tick-borne encephalitis in Europe and Russia: Review of pathogenesis, clinical features, therapy, and vaccines. *Antiviral Res.* **2019**, *164*, 23–51. [CrossRef] [PubMed]
2. Beauté, J.; Spiteri, G.; Warns-Petit, E.; Zeller, H. Tick-Borne Encephalitis in Europe, 2012 to 2016. *Eurosurveillance* **2018**, *23*. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6234529/ (accessed on 10 July 2023). [CrossRef] [PubMed]
3. European Centre for Disease Prevention and Control. Tick-Borne Encephalitis. Annual Epidemiological Report for 2020. Stockhom: ECDC. Available online: https://www.ecdc.europa.eu/en/publications-data/tick-borne-encephalitis-annual-epidemiological-report-2020 (accessed on 10 July 2023).
4. Salat, J.; Ruzek, D. Tick-borne encephalitis in domestic animals. *Acta Virol.* **2020**, *64*, 226–232. [CrossRef]
5. Kleeb, C.; Golini, L.; Beckmann, K.; Torgerson, P.; Steffen, F. Canine Tick-Borne Encephalitis: Clinical Features, Survival Rate and Neurological Sequelae: A Retrospective Study of 54 Cases (1999–2016). *Front. Vet. Sci.* **2021**, *8*, 782044. [CrossRef]
6. Levanov, L.; Vera, C.P.; Vapalahti, O. Prevalence estimation of tick-borne encephalitis virus (TBEV) antibodies in dogs from Finland using novel dog anti-TBEV IgG MAb-capture and IgG immunofluorescence assays based on recombinant TBEV subviral particles. *Ticks Tick-Borne Dis.* **2016**, *7*, 979–982. [CrossRef]
7. Lindhe, K.E.; Meldgaard, D.S.; Jensen, P.M.; Houser, G.A.; Berendt, M. Prevalence of tick-borne encephalitis virus antibodies in dogs from Denmark. *Acta Vet. Scand.* **2009**, *51*, 56. [CrossRef]
8. Reiner, B.; Grasmück, S.; Steffen, F.; Djuric, N.; Schindler, T.; Müller, W.; Fischer, A. Prevalence of TBE antibodies in serum and CSF of dogs with inflammatory and non-inflammatory CNS disease. *Int. J. Med. Microbiol.* **2002**, *291*, 234. [CrossRef]
9. Pfeffer, M.; Dobler, G. Tick-borne encephalitis virus in dogs—Is this an issue? *Parasit. Vectors.* **2011**, *4*, 59. [CrossRef]
10. Leschnik, M.W.; Kirtz, G.C.; Thalhammer, J.G. Tick-borne encephalitis (TBE) in dogs. *Int. J. Med. Microbiol.* **2002**, *291*, 66–69. [CrossRef]
11. Dultz, R.; Goldhammer, M. Frühsommer-Meningoenzephalitis bei einem Hund. *Tierärztl. Prax. Ausg. K Kleintiere Heimtiere* **2021**, *49*, 377–381. [CrossRef]
12. Bogovic, P. Tick-borne encephalitis: A review of epidemiology, clinical characteristics, and management. *World J. Clin. Cases* **2015**, *3*, 430. [CrossRef] [PubMed]
13. Bogovic, P.; Lotric-Furlan, S.; Strle, F. What tick-borne encephalitis may look like: Clinical signs and symptoms. *Travel. Med. Infect. Dis.* **2010**, *8*, 246–250. [CrossRef]
14. Andersson, E.; Kendall, A.; Url, A.; Auer, A.; Leschnik, M. The first RT-qPCR confirmed case of tick-borne encephalitis in a dog in Scandinavia. *Acta Vet. Scand.* **2020**, *62*, 51. [CrossRef]
15. Bajer, A.; Rodo, A.; Bednarska, M.; Mierzejewska, E.; Welc-Falęciak, R. Babesia canis and tick-borne encephalitis virus (TBEV) co-infection in a sled dog. *Ann. Agric. Environ. Med.* **2013**, *20*, 426–430. [PubMed]
16. Vilibic-Cavlek, T.; Ferenc, T.; Vujica Ferenc, M.; Bogdanic, M.; Potocnik-Hunjadi, T.; Sabadi, D.; Savic, V.; Barbic, L.; Stevanovic, V.; Monaco, F.; et al. Cross-Reactive Antibodies in Tick-Borne Encephalitis: Case Report and Literature Review. *Antibodies* **2022**, *11*, 72. [CrossRef] [PubMed]
17. Holzmann, H. Diagnosis of tick-borne encephalitis. *Vaccine* **2003**, *21*, S36–S40. [CrossRef]
18. Holzmann, H.; Kundi, M.; Stiasny, K.; Clement, J.; McKenna, P.; Kunz, C.; Heinz, F.X. Correlation between ELISA, hemagglutination inhibition, and neutralization tests after vaccination against tick-borne encephalitis. *J. Med. Virol.* **1996**, *48*, 102–107. [CrossRef]

19. Puchhammer-Stöckl, E.; Kunz, C.; Mandl, C.W.; Heinz, F.X. Identification of tick-borne encephalitis virus ribonucleic acid in tick suspensions and in clinical specimens by a reverse transcription-nested polymerase chain reaction assay. *Clin. Diagn. Virol.* **1995**, *4*, 321–326. [CrossRef]
20. Hekrlová, A.; Kubíček, O.; Lány, P.; Rosenbergová, K.; Schánilec, P. Tick-borne encephalitis in dogs: Application of "nested real-time RT-PCR" for intravital virus detection. *Berl. Munch. Tierarztl. Wochenschr.* **2015**, *128*, 397–401.
21. Beckmann, K.; Steffen, F.; Ohlerth, S.; Kircher, P.R.; Carrera, I. Three Tesla Magnetic Resonance Imaging Findings in 12 Cases of Canine Central European Tick-borne Meningoencephalomyelitis. *Vet. Radiol. Ultrasound.* **2016**, *57*, 41–48. [CrossRef]
22. Salat, J.; Hunady, M.; Svoboda, P.; Strelcova, L.; Strakova, P.; Fortova, A.; Palus, M.; Ruzek, D. Efficacy and immunogenicity of a veterinary vaccine candidate against tick-borne encephalitis in dogs. *Vaccine* **2023**, *41*, 6150–6155. [CrossRef] [PubMed]
23. Saksida, A.; Duh, D.; Lotrič-Furlan, S.; Strle, F.; Petrovec, M.; Avšič-Županc, T. The importance of tick-borne encephalitis virus RNA detection for early differential diagnosis of tick-borne encephalitis. *J. Clin. Virol.* **2005**, *33*, 331–335. [CrossRef]
24. Pautienius, A.; Armonaite, A.; Simkute, E.; Zagrabskaite, R.; Buitkuviene, J.; Alpizar-Jara, R.; Grigas, J.; Zakiene, I.; Zienius, D.; Salomskas, A.; et al. Cross-Sectional Study on the Prevalence and Factors Influencing Occurrence of Tick-Borne Encephalitis in Horses in Lithuania. *Pathogens* **2021**, *10*, 140. [CrossRef]
25. Kirz, G.; Kirtz, G. Frühsommer-Meningo-Encephalitis (FSME) in Einer Österreichischen Hundepopulation. Ph.D. Thesis, Veterinärmedizinische Universität Wien, Vienna, Austria, 1999.
26. Salat, J.; Hunady, M.; Schanilec, P.; Strakova, P.; Stefanik, M.; Svoboda, P.; Strelcova, L.; Bojcukova, J.; Palus, M.; Růžek, D. Experimental and Natural Infections of Tick-Borne Encephalitis Virus in Dogs. *Viruses* **2021**, *13*, 2039. [CrossRef] [PubMed]
27. Hellenbrand, W.; Kreusch, T.; Böhmer, M.; Wagner-Wiening, C.; Dobler, G.; Wichmann, O.; Altmann, D. Epidemiology of Tick-Borne Encephalitis (TBE) in Germany, 2001–2018. *Pathogens* **2019**, *8*, 42. [CrossRef] [PubMed]
28. Lenhard, T.; Ott, D.; Jakob, N.J.; Pham, M.; Bäumer, P.; Martinez-Torres, F.; Meyding-Lamadé, U. Predictors, Neuroimaging Characteristics and Long-Term Outcome of Severe European Tick-Borne Encephalitis: A Prospective Cohort Study. *PLoS ONE* **2016**, *11*, e0154143. [CrossRef] [PubMed]
29. Radzišauskienė, D.; Žagminas, K.; Ašoklienė, L.; Jasionis, A.; Mameniškienė, R.; Ambrozaitis, A.; Jančorienė, L.; Jatužis, D.; Petraitytė, I.; Mockienė, E. Epidemiological patterns of tick-borne encephalitis in Lithuania and clinical features in adults in the light of the high incidence in recent years: A retrospective study. *Eur. J. Neurol.* **2018**, *25*, 268–274. [CrossRef]
30. Kleiter, I.; Jilg, W.; Bogdahn, U.; Steinbrecher, A. Delayed Humoral Immunity in a Patient with Severe Tick-borne Encephalitis after Complete Active Vaccination. *Infection* **2007**, *35*, 26–29. [CrossRef]
31. Roelandt, S.; Suin, V.; Gucht, S.V.; Stede, Y.V.; Roels, S. Comparative Tick-Borne Encephalitis (Virus) Surveillance in Belgium 2009-2015: Experiences with Diagnostic Tests, Sentinel Species and Surveillance Designs. *J. Zoonotic. Dis. Public Health* **2017**, *1*, 4. Available online: https://www.imedpub.com/articles/comparative-tickborne-encephalitisvirus-surveillance-in-belgium-20092015experiences-with-diagnostic-tests-sentinelspecies-and-surv.php?aid=19858 (accessed on 25 October 2023).
32. Stadtbaumer, K.; Leschnik, M.W.; Nell, B. Tick-borne encephalitis virus as a possible cause of optic neuritis in a dog. *Vet. Ophthalmol.* **2004**, *7*, 271–277. [CrossRef]
33. Sioutas, G.; Tsakou, K.; Top, C.; Jongejan, F.; Papadopoulos, E. First clinical case of tick-borne encephalitis (TBE) in a dog in Greece. *Ticks Tick-borne Dis.* **2023**, *14*, 102226. [CrossRef] [PubMed]
34. Soleng, A.; Edgar, K.S.; Paulsen, K.M.; Pedersen, B.N.; Okbaldet, Y.B.; Skjetne, I.E.B.; Gurung, D.; Vikse, R.; Andreassen, Å.K. Distribution of Ixodes ricinus ticks and prevalence of tick-borne encephalitis virus among questing ticks in the Arctic Circle region of northern Norway. *Ticks Tick-Borne Dis.* **2018**, *9*, 97–103. [CrossRef] [PubMed]
35. Zubriková, D.; Wittmann, M.; Hönig, V.; Švec, P.; Víchová, B.; Essbauer, S.; Dobler, G.; Grubhoffer, L.; Pfister, K. Prevalence of tick-borne encephalitis virus and Borrelia burgdorferi sensu lato in Ixodes ricinus ticks in Lower Bavaria and Upper Palatinate, Germany. *Ticks Tick-Borne Dis.* **2020**, *11*, 101375. [CrossRef] [PubMed]
36. Wójcik-Fatla, A.; Cisak, E.; Zając, V.; Zwoliński, J.; Dutkiewicz, J. Prevalence of tick-borne encephalitis virus in Ixodes ricinus and Dermacentor reticulatus ticks collected from the Lublin region (eastern Poland). *Ticks Tick-Borne Dis.* **2011**, *2*, 16–19. [CrossRef] [PubMed]
37. Belova, O.A.; Burenkova, L.A.; Karganova, G.G. Different tick-borne encephalitis virus (TBEV) prevalences in unfed versus partially engorged ixodid ticks—Evidence of virus replication and changes in tick behavior. *Ticks Tick-Borne Dis.* **2012**, *3*, 240–246. [CrossRef] [PubMed]
38. Lindblom, P.; Wilhelmsson, P.; Fryland, L.; Sjöwall, J.; Haglund, M.; Matussek, A.; Ernerudh, J.; Vene, S.; Nyman, D.; Andreassen, A.; et al. Tick-borne encephalitis virus in ticks detached from humans and follow-up of serological and clinical response. *Ticks Tick-Borne Dis.* **2014**, *5*, 21–28. [CrossRef]
39. Bormane, A.; Lucenko, I.; Duks, A.; Mavtchoutko, V.; Ranka, R.; Salmina, K.; Baumanis, V. Vectors of tick-borne diseases and epidemiological situation in Latvia in 1993–2002. *Int. J. Med. Microbiol. Suppl.* **2004**, *293*, 36–47. [CrossRef]
40. Korenberg, E.I.; Gorban, L.Y.; Kovalevskii, Y.V.; Frizen, V.I.; Karavanov, A.S. Risk for Human Tick-Borne Encephalitis, Borrelioses, and Double Infection in the Pre-Ural Region of Russia. *Emerg. Infect. Dis.* **2001**, *7*, 459–462. [CrossRef]

41. Zambito Marsala, S.; Pistacchi, M.; Gioulis, M.; Mel, R.; Marchini, C.; Francavilla, E. Neurological complications of tick-borne encephalitis: The experience of 89 patients studied and literature review. *Neurol. Sci.* **2014**, *35*, 15–21. [CrossRef]
42. Mickienė, A.; Laiškonis, A.; Günther, G.; Vene, S.; Lundkvist, Å.; Lindquist, L. Tickborne Encephalitis in an Area of High Endemicity in Lithuania: Disease Severity and Long-Term Prognosis. *Clin. Infect. Dis.* **2002**, *35*, 650–658. [CrossRef]

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Article

The Prevalence of Tick-Borne Encephalitis Virus in Wild Rodents Captured in Tick-Borne Encephalitis Foci in Highly Endemic Lithuania

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Abstract: Wild rodents are considered to be one of the most important TBEV-amplifying reservoir hosts; therefore, they may be suitable for foci detection studies. To investigate the effectiveness of viral RNA detection in wild rodents for suspected TBEV foci confirmation, we trapped small rodents (n = 139) in various locations in Lithuania where TBEV was previously detected in questing ticks. Murine neuroblastoma Neuro-2a cells were inoculated with each rodent sample to maximize the chances of detecting viral RNA in rodent samples. TBEV RNA was detected in 74.8% (CI 95% 66.7–81.1) of the brain and/or internal organ mix suspensions, and the prevalence rate increased significantly following sample cultivation in Neuro-2a cells. Moreover, a strong correlation (r = 0.88; $p < 0.05$) was found between the average monthly air temperature of rodent trapping and the TBEV RNA prevalence rate in cell culture isolates of rodent suspensions, which were PCR-negative before cultivation in cell culture. This study shows that wild rodents are suitable sentinel animals to confirm TBEV foci. In addition, the study results demonstrate that sample cultivation in cell culture is a highly efficient method for increasing TBEV viral load to detectable quantities.

Keywords: tick-borne encephalitis; TBEV RNA prevalence; wild rodents; cell culture

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1. Introduction

Tick-borne encephalitis (TBE) is the most common and medically important tick-borne viral zoonosis in Europe and Northern Asia [1]. In Lithuania, the TBE infection rate has been the highest in Europe for nearly 10 years [2]. Although TBE is typically characterized as a focal infection, the whole country of Lithuania is considered to be TBEV-endemic [3].

The tick-borne encephalitis virus (TBEV) maintains its cycle by circulating between vector ticks and reservoir animals [4]. Although the exact mechanism of focal TBEV distribution in nature is still unclear, interactions between ticks and reservoir hosts of the virus, as well as particular environmental and climatic factors, are considered to be the most influential [5–7]. A study conducted by Daniel et al. [8] suggests that warm climatic conditions might be related to a higher TBEV load in ticks in the summer and autumn periods and, therefore, to an increased risk of human TBE cases. Moreover, an increased rate of outdoor activities in potentially tick-infested areas in summer and autumn might be related to a higher incidence of human TBE cases [9,10].

Ticks were analyzed for viral RNA presence in the majority of TBEV foci detection studies, although the reported TBEV prevalence rate in ticks was primarily low. Further-

more, a large sample size of ticks must be analyzed to detect TBEV, and tick collection is a time-consuming and labor-intensive task fraught with the risk of tick bites and tick-borne infections [11]. Domestic animals of various species, including dogs, horses, goats, sheep, and wild animals such as rodents, insectivores, roe deer, wild boar, and wild birds, have all been investigated for TBEV seroprevalence and the suitability of TBEV foci monitoring [12–14].

To date, only small mammals, such as some species of rodents and insectivores, are suspected to be sufficient reservoir hosts suitable for virus amplification and maintenance [13]. Few studies have shown a correlation between TBEV seroprevalence in rodents and local incidence of human TBE [15,16]. Small rodents might be useful sentinels for TBEV foci detection and monitoring since they tend to be highly infested with ticks, do not migrate long distances, tend to populate in high quantities, and are convenient to trap and collect [15]. Moreover, studies in highly endemic Siberian regions suggest that in endemic areas, the majority of wild rodents might be persistently infected with TBEV [17]. Although the majority of TBEV research in Europe was focused on detecting TBEV-specific antibodies in wild rodents, some authors suggest that the TBEV-Eu subtype can persist in naturally infected rodents without detectable antibodies [18,19].

The present study aimed to investigate the prevalence rate of TBEV RNA in wild rodents in TBE foci in Lithuania. Based on data suggesting persistent TBE infection in rodents, we hypothesized that cultivation of rodent brain and internal organ mix suspensions in murine neuroblastoma Neuro-2a cell culture could increase the viral load to a detectable level and possibly provide more insight into TBEV prevalence and maintenance in wild rodents.

2. Materials and Methods

2.1. Sample Collection and Sampling Sites

Wild rodents were collected in 19 endemic locations (5 districts) in Lithuania. Specific sites for rodent trapping were selected according to the National Public Health Center under the Ministry of Health, which has provided a complete map of confirmed human TBE cases with probable TBEV-infected tick bite locations or known TBEV foci from 2016 to 2018 (https://nvsc.lrv.lt/lt/uzkreciamuju-ligu-valdymas/uzkreciamosios-ligos/erkiu-pernesamos-ligos/lietuvos-vietoviu-kuriose-uzsikreciama-erkiu-platinamomis-ligomis-zemelapis/ (accessed on 27 February 2024). Moreover, TBEV was detected in questing ticks in the majority of rodent trapping sites in a previous study [20]. Small rodents (n = 139) were trapped in different TBEV foci from March 2019 until May 2020, excluding the summer months when trapping was not productive. Rodents were caught in live traps and presented to the laboratory on the same day. The species, sex, and size of the animals were determined, and necropsy was performed according to standard protocols. Brain, spleen, liver, heart, kidneys, and 5 fetal samples were collected and stored at –80 °C until further analysis. The data on average monthly air temperature was obtained from annual reports of the Lithuanian Hydrometeorological Service under the Ministry of Environment (https://www.meteo.lt/category/menesio-hidrometeorologiniu-salygu-apzvalga/page/5/ (accessed on 27 February 2024). The trapping of rodents was performed after confirmation by the Ministry of Environment of the Republic of Lithuania with license No. 5 (28 February 2019) and No. 12 (27 February 2020) according to animal welfare regulations.

2.2. Detection of TBEV RNA and Viral Load Quantification

Pieces (10–100 µg) of the brain (n = 137) and internal organs such as the spleen, liver, heart, and kidneys of each rodent (n = 139) were homogenized separately using a mortar and pestle, then mixed with 1000 µL of Modified Eagle's Medium (MEM, Gibco, Waltham, MA, USA) and stored at –80 °C for further analysis. Brain samples were analyzed separately from the internal organ mix samples obtained from the same rodent. Total RNA was extracted from 300 µL homogenate with GeneJET RNA Purification Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. For the presence of TBEV RNA, extracted samples were analyzed by real-time reverse transcription polymerase

chain reaction (RT-PCR) using a reaction mix containing SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Thermo Scientific, Waltham, MA, USA) and primers as described previously [21]. Selected RT-PCR-positive samples were screened by nested PCR using primers and DreamTaq Green PCR Master Mix as described previously [22]. PCR-positive samples were confirmed by partial genome sequencing targeting the 126 bp NCR region of TBEV using the same primer set according to a previous study [21].

The viral quantification assay was modified according to previous studies [21,23]. Briefly, a synthetic fragment corresponding to the amplified TBEV NS5 region was cloned into the pJET1.2 vector using the CloneJET PCR Cloning Kit (Thermo Scientific, Waltham, MA, USA) and transformed into pretreated *Escherichia coli* cells using the Transform-Aid Bacterial Transformation Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Transformed *E. coli* was cultivated overnight at 37 °C. Plasmid DNA was extracted and purified using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, Waltham, MA, USA) and quantified using the Qubit dsDNA BR Assay Kit (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. Standard curves were generated after 10-fold dilutions of stock DNA, which served as templates for qPCR reactions. Sample concentration was calculated using a calibrated standard curve, and viral load was estimated using a quantification assay. Quantification was based on RT-PCR using SYBR Green I Dye (Thermo Scientific, Waltham, MA, USA) and nested NS5 primers as described previously [21]. All samples were tested in triplicates, and mean values were calculated.

2.3. Virus Isolation

For cell culture inoculation, 500 µL of homogenate was centrifuged at 12,000× *g* for 10 min and filtered with a 0.22 µm pore size microfilter (Techno Plastic Products AG, Trasadingen, Switzerland). Murine neuroblastoma cells (Neuro-2a ATCC No. CCL-131) were seeded in 96-well plates and incubated in a maintenance medium containing ½ Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) and ½ Modified Eagle's Medium (MEM, Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in 5% CO_2. The following day, cells were inoculated for 1 h with 100 µL of microfiltered internal organ mix supernatant and brain supernatant. Cells were then washed with PBS and incubated at 37 °C in 5% CO_2 in 200 µL of Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA) and 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were inoculated in triplicate for each sample, incubated for 4 days, and monitored for occurrence of cytopathic effect. Following incubation, cells were frozen at –40 °C, thawed two times, and centrifugated at 12,000× *g* for 10 min before the next inoculation. After 3 serial passages, cells were frozen and thawed two times, and the cell suspension was harvested for RNA extraction. Successful virus cultivation was confirmed by RT-PCR.

2.4. Statistical Analysis

The exact binomial method was used to calculate confidence intervals. Binary logistic regression analysis, Chi-square test, and Fisher's exact test were utilized to test the significance of the risk factors. Linear associations were assessed by the Pearson correlation test. All statistical analysis and mapping were performed using the programming language R.

3. Results

3.1. TBEV RNA Prevalence in Wild Rodents

In total, 137 brain and 139 internal organ mix homogenates from 139 wild rodents were investigated by RT-PCR. TBEV RNA was detected in 104 (74.8%; CI 95% 66.7–81.1) wild rodents. Brain suspensions of 51.1% (CI 95% 42.4–59.7) and internal organ mix suspensions of 53.2% (CI 95% 44.6–61.7) rodents were positive for viral RNA. Further-

more, 28.8% (CI 95% 21.4–37.1) of both brain and internal organ mix suspensions of the same animal were positive for viral RNA. The specificity of the obtained PCR-positive samples was confirmed by partial genome sequencing targeting the 126 bp NCR region (Supplementary Materials Figure S1).

The viral RNA detection rate increased significantly after cultivation in Neuro-2a cell culture (Figure 1), resulting in a total of 134 (96.4%; CI 95% 91.8–98) positive rodents. Brain isolates of 85.4% (CI 95% 78.4–90.9) and internal organ mix isolates of 81.3% (CI 95% 73.8–87.4) rodents were positive for viral RNA. In 69.1% (CI 95% 60.7–76.6) of rodents, TBEV-specific RNA was concurrently found in both types of samples. Moreover, TBEV was detected in both types of rodent samples (n = 30), which were PCR-negative before sample cultivation in cell culture.

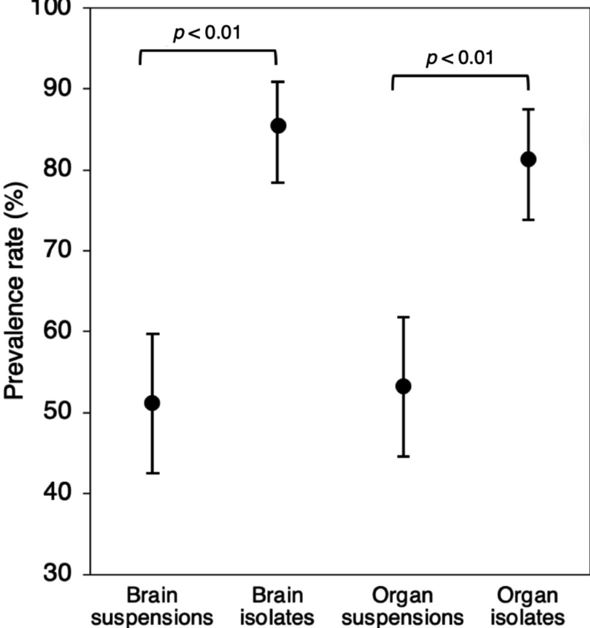

Figure 1. TBEV RNA prevalence rate in wild rodent brain and internal organ mix samples before (suspensions) and after (isolates) cultivation in murine neuroblastoma (Neuro-2a) cell line.

In addition, viral genome was detected in two out of five fetus suspension samples and in all five samples after isolation in cell culture. In four out of five cases, viral RNA was not detected in relative maternal brain and internal organ suspension samples before isolation in cell culture.

Viral RNA was detected in at least one of the brain or internal organs mix suspension samples of rodents collected in all 19 endemic locations of our study (Figure 2). Based on PCR results from suspension samples, infection rates at the spatial scale ranged from 25 to 100% (Figure 2A). The prevalence rate following sample cultivation in cells increased to 100% in 16 trapping locations, and the adjusted prevalence rate was 71.4–90% in the remaining 3 locations (Figure 2B).

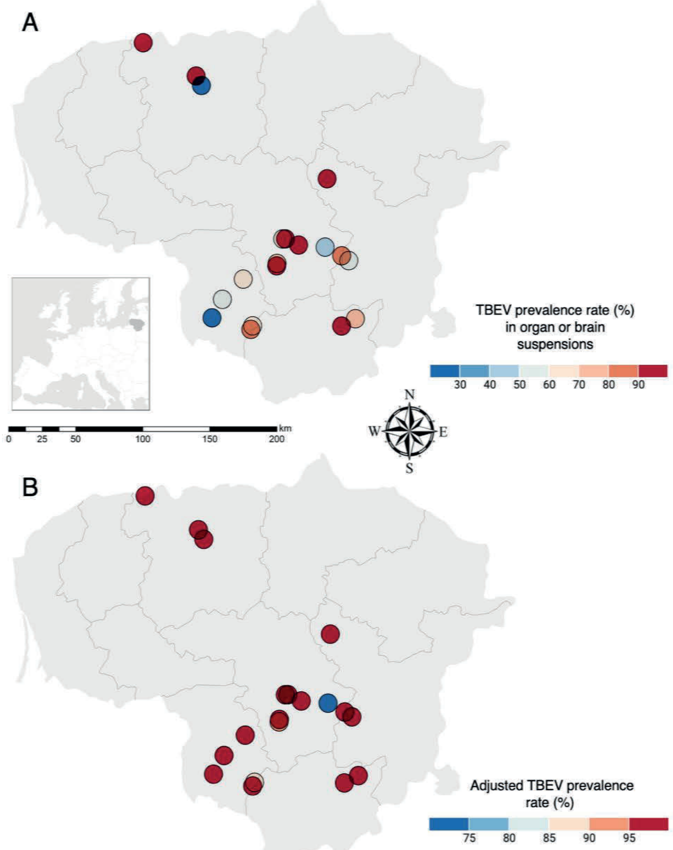

Figure 2. (**A**) Spatial distribution of sample collection sites and average TBEV RNA prevalence rate before isolation and (**B**) after isolation in Neuro-2a cells in different trapping locations in Lithuania. Viral RNA prevalence rate (%) in wild rodents is indicated by different colors shown in legends.

3.2. Comparison of TBEV Viral Load in Suspensions and Neuro-2a Cell Isolates

Viral load in the majority of brain and internal organ mix suspension samples was low, and in 70.1% of TBEV-positive suspension samples, amplification was observed at threshold cycle (Ct) values of 35–40. Brain and internal organ mix suspension samples ($n = 30$) with the lowest Ct value and respective rodent sample isolates in cells ($n = 30$) were taken, and viral load was measured (Figure 3). The average viral load in the same rodent's Neuro-2a cell culture isolates was significantly higher ($p < 0.05$) than in the respective tissue suspension samples (6.4 $\log_{10}$ copies/mL and 5.7 $\log_{10}$ copies/mL, respectively). The results demonstrate successful TBEV isolation in cell culture and confirm viable virus presence in rodent tissue samples.

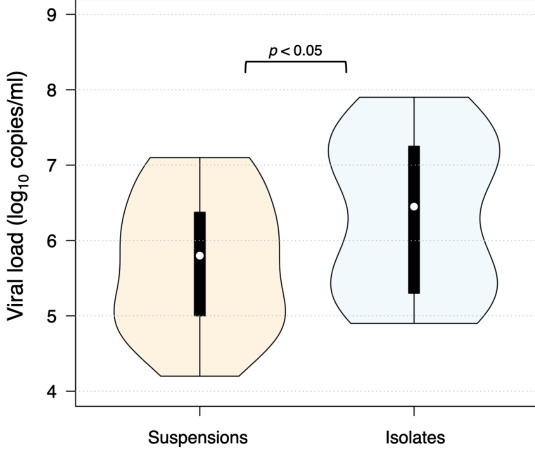

Figure 3. TBEV viral load in selected rodent brain and internal organ mix samples (n = 30) before (suspensions) and after (isolates) isolation in murine neuroblastoma Neuro-2a cells.

3.3. Seasonal Trends of TBEV Prevalence in Rodents

Rodent samples were collected when the average air temperature was below 10 °C, and tick activity was not at its peak. The prevalence rate of TBEV RNA in rodent suspension samples from all collection sites was analyzed when grouped according to the month of trapping (Supplementary Materials Table S1).

A strong correlation (r = 0.88; $p < 0.05$) was found between average monthly air temperature and TBEV RNA prevalence rate in cell culture isolates of rodent suspensions, which were PCR-negative before cultivation (Figure 4). The detected viral RNA prevalence rate in isolates of brain and internal organ mix samples, which were PCR-negative prior to cultivation, was significantly higher in rodents trapped when the average monthly air temperature was higher than 5 °C.

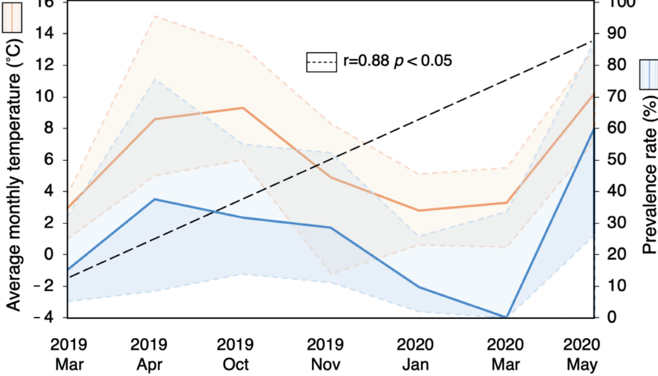

Figure 4. Longitudinal distribution of the prevalence of TBEV RNA in rodent brain and internal organ mix suspension samples that were PCR-negative before isolation in Neuro-2a cell line and the average air temperature of the rodent trapping month.

3.4. TBEV Prevalence in Different Rodent Species

Overall, rodents of six species were trapped and investigated in the study: 76 *Apodemus flavicollis*, 29 *Clethrionomys glareolus*, 22 *Microtus arvalis*, 9 *Mus musculus*, 2 *Apodemus sylvaticus*, and 1 *Apodemus agrarius*.

TBEV RNA-positive brain and/or internal organ mix suspension samples were detected in 77.6% (59/76) *A. flavicollis*, 72.4% (21/29) *C. glareolus*, 100% (22/22) *M. arvalis*, 75% (6/9) *M. musculus*, 100% (2/2) *A. sylvaticus*, and 100% (1/1) *A. agrarius*. The prevalence rate of viral RNA in brain and internal organ mix suspension samples was found to be significantly higher ($p < 0.05$) in *M. arvalis* rodents compared to other species. On the other hand, the prevalence of TBEV RNA in suspension samples of *A.flavicollis* was significantly lower compared to other rodent species (Fisher's exact test $p = 0.46$). However, after cultivation in Neuro-2a cells, no significant differences between rodent species and RNA prevalence rate were found. Only 5 of 139 rodents were negative for TBEV RNA, and all of them belonged to *A. flavicollis* species (Supplementary Materials Table S2).

Five fetal samples were investigated in the study (not included in the overall rodent count). Viral RNA in *M. arvalis* was detected in both maternal samples and fetal samples before and after cultivation in cell culture. In *C. glareolus*, viral RNA was detected only in maternal suspensions before cultivation in neuroblastoma cells. Notably, TBEV RNA was found in one of the *A. flavicollis* fetal suspension samples but not in the respective maternal suspension samples. In the remaining samples of two *A. flavicollis* rodents, viral RNA in both maternal samples and fetal samples was detected only after cultivation in cell culture.

4. Discussion

Our study revealed that in confirmed TBEV foci in Lithuania, viral RNA can be found in the majority of wild rodent brain and internal organ mix samples. TBEV RNA was present in 74.8% of rodent brain and internal organ mix suspensions and 96.4% of rodent samples after sample cultivation in neuroblastoma cells. It is the highest reported TBEV RNA prevalence rate in wild rodents, although reports on TBEV-Eu genome presence in wild rodents are scarce. A study performed in high-risk areas in Hungary detected a TBEV RNA prevalence rate of 4.2% (17/405) in rodent liver samples collected over 7 years [24]. In Germany, viral RNA was found in 15% (21/137) of rodent brain and spleen samples in TBEV-risk areas and 8% (24/304) of rodents captured in non-risk areas [15]. A study conducted by Tonteri et al. [18] in two separate TBEV-Sib and TBEV-Eu endemic areas in Finland reported a TBEV RNA prevalence rate of 16.8% (16/95) in TBEV-Eu endemic zone-collected and 6.3% (5/80) in TBEV-Sib endemic zone-collected wild rodents trapped in late winter. Moreover, the reported prevalence was significantly higher in 2009 compared to 2008 (54.2% (13/24) TBEV-Eu and 23.5% (4/17) TBEV-Sib in 2009 vs. 4.2% (3/71) TBEV-Eu and 1.6% (1/63) TBEV-Sib in 2008), although the sampling sites, rodent species, and trapping time were the same in both years of trapping [18]. In endemic areas of Siberia, authors reported a high TBEV-Sib RNA prevalence rate in wild rodents and insectivores, reaching 61–74% [17,25]. Although the data on TBEV RNA prevalence in wild rodents are scarce, the variety of prevalence rates in endemic areas is high, which indicates that many factors might play a role in affecting the prevalence rate.

Several factors might have contributed to the high TBEV prevalence rate in rodents revealed in our study. The human TBE infection rate in Lithuania is the highest in Europe and considerably higher compared to other European countries where TBEV RNA was detected in rodents [2]. Moreover, specific rodent trapping sites were chosen according to the data of detected TBEV-positive ticks, possible tick bite locations, and related human TBE cases. As concluded by Borde et al. [26], the TBEV transmission cycle occurs in small areas (average size of about 0.5–1 ha) of so-called microfoci, which are stable for decades and usually do not expand or shift. Although the TBEV transmission cycles and their stability in microfocus are still not understood, and so far, environmental models do not provide possible explanations [26], capturing rodents in the specific areas of microfocus might increase the chances of TBEV detection. Furthermore, a greater variety of organ

tissues were taken for RNA extraction, and brain and internal organ mix samples of each rodent were analyzed separately by RT-PCR. In addition, a landscape of Lithuania rich in agricultural fields, woodlands, and forests, together with humid climatic conditions, is preferable for ticks and might be related to higher tick density. A substantial part of city parks and green zones are also known as TBEV foci, and the retrospective epidemiological study revealed that 60% of TBE patients were inhabitants of cities and 42% were infected in the living area, which shows high TBE risk even in urban areas [27,28]. Moreover, the agricultural landscape is important for the sustainability of the rodent population of some species, and a low trapped rodent number from late spring to early autumn might be related to food abundance in fields. Furthermore, agricultural fields attract deer, which are important tick hosts and have been suggested to be related to the increased risk of human TBE [29,30]. A recent study conducted in Lithuania revealed that the deer population has been exponentially increasing in the past 20 years [31].

Previous studies in Lithuania showed a low TBEV prevalence rate in field-collected ticks. According to various studies, the reported minimal infection rate (MIR) in ticks ranged from 0.1 to 1.8% [20,32,33]. Nevertheless, recent studies confirmed widespread TBEV distribution in the country based on the prevalence rate of antibodies against TBEV in horses (37.5%) and virus presence in horse serum samples (3.9%), as well as in goat (4.3%) and sheep (4.5%) milk samples collected in endemic localities in Lithuania [34,35]. Moreover, in a recent study, TBEV-specific antibodies were detected in 21.6% and TBEV RNA in 18.6% of randomly collected blood serum samples of dogs residing in the second-largest city in Lithuania. Although the vital virus was isolated in cell culture only from a small portion of PCR-positive dog samples, mainly due to the limited volume of obtained blood serum samples, TBEV was isolated from ticks collected from the dogs. Similar to the present study, the TBEV RNA prevalence rate in ticks increased significantly after sample isolation in cell culture (34.2% before vs. 56.4–60.7% after cultivation in different cell cultures) [36].

To the best of our knowledge, no previous studies have attempted to isolate wild rodent-derived TBEV in murine neuroblastoma cells to investigate the virus prevalence rate. A study with a similar goal was performed in an attempt to isolate TBEV in chicken embryo cell culture from *A. flavicollis* blood samples, although it was unsuccessful [37]. After sample cultivation in murine Neuro-2a cells, the detection rate of TBEV RNA and, thus, the overall prevalence rate increased significantly ($p < 0.01$). Successful virus isolation from PCR-negative rodent samples demonstrated that murine neuroblastoma cells are highly susceptible to rodent-derived TBEV infection. Cell lines of neural origin were found to be highly susceptible to TBEV infection, providing 100- to 10,000-fold higher virus titer than cells of non-neural origin [38]. Experimental studies show that virus adaptation to a specific cell line necessitates serial passages for structural rearrangements that favor viral load increasing to detectable levels [39]. As a result, compatible viral host and cell line origin may have played a significant role in high virus isolation success.

The majority of tissue suspension samples were positive for TBEV RNA, albeit characterized by high Ct values, therefore indicating low viral loads. This finding is in line with previous studies that found low viral copy levels in brain and spleen samples of wild rodents [13,15]. Low viral load in the majority of wild rodents might be due to the longevity of TBE infection. Persisting TBEV was found in the brains of various small rodents and insectivores in several investigations [15,17,18,40,41]. A naturally persistent infection in *Clethrionomys rutilus* was demonstrated to last for up to 10 months [42]. However, we have successfully isolated viable TBE virus in murine neuroblastoma cells from rodent samples that were positive at late RT-PCR cycles and from samples ($n = 30$) that were PCR-negative. Michelitsch et al. [41] successfully isolated viable TBEV in human lung carcinoma A549 cells from experimentally infected bank vole brain and internal organ samples that showed low Ct values (<36.0). The success of virus isolation was related to different TBEV strains. In comparison to the reference Neudörfl strain, the more recently isolated TBEV strains were more effectively reisolated in cells, indicating that virus strain plays a role in successful

isolation in cell culture [41]. Moreover, although PCR is considered a highly specific and sensitive method for TBEV RNA detection, a study conducted by Donoso Mantke et al. [43] revealed significant limitations in reliably detecting the virus. Only 2 out of 23 laboratories that took part in the study found TBEV in all of the provided human blood serum samples that had different strains and amounts of the virus [43].

A strong correlation was found between average monthly air temperature and TBEV RNA prevalence rate in cell culture isolates of rodent suspension samples that were PCR-negative before cultivation. TBEV RNA was detected more often in rodents captured at a lower average air temperature than in rodents trapped in warmer weather. When the average air temperature of rodent trapping increased, it led to a lower RNA detection rate in suspensions and an increased detection rate in cell culture isolates of the same rodent samples. It is worth noting that the warmest January since 1961 at the time of sample collection, as well as an exceptionally cold May in Lithuania, might have influenced the findings. To the best of our knowledge, no previous study reported a similar trend. A study with known rodent trapping time conducted in Finland found a higher TBEV-Eu RNA prevalence rate in samples collected during colder winter (54.2%) compared to a warmer one (4.2%), even though the collecting area, trapping month, and rodent species were the same. Moreover, the authors reported a high TBEV-Eu RNA prevalence rate in wild rodents trapped in a specific TBEV focus in late winter, when ticks were not active for a few months. In addition, TBEV-specific antibodies were detected in only 12.5% (2/16) of TBEV-Eu RNA-positive rodents and in 100% (5/5) of TBEV-Sib RNA-positive rodents, which indicates that the majority of TBEV-Eu-infected rodents might not have detectable antibodies in persistent TBE infection [18].

The influence of air temperature on TBEV replication was observed in previous studies on hedgehogs (*Erinaceus roumanicus*). According to Nosek and Grulich [44], the duration of viremia in hedgehogs is indirectly proportional to air temperature, lasting 3–6 days in warm summer and 8–14 days in spring and autumn. Additionally, intense and long-lasting viremia can persist throughout the hibernation period in infected mammals [44]. The opposite trend was suggested in ticks: higher air temperature is associated with increased human TBE incidence rate and potentially higher virus load in ticks [8,45]. A study conducted in the Czech Republic reported that most human TBE cases were recorded when the air temperature was 10–20 °C [46]. Moreover, Korenberg and Kovalevskii [47] suggested that the incidence of clinical TBE most closely corresponds to the actual probability of being bitten by a highly infected tick. Furthermore, a thermosensitive RNA switch has been proposed as significant for virus propagation in ticks [48]. However, other factors such as duration of photoperiod, relative humidity [8,26], age and sex [49], reproductive and immune status [50], and tick burden load on rodents [51], which are also at least partially related to the average air temperature, might be influential. Moreover, a rapid fall in ground-level temperatures in August and October is associated with the synchrony of larval-nymphal questing (and therefore co-feeding) in early spring, which was observed in TBEV foci and not elsewhere [6]. However, to date, there is no robust ecological evidence that co-feeding is important for the stability of TBEV cycles in nature [5].

Early spring is a favorable time for TBEV activation from a latent state in rodent organisms due to a weakened immune response during winter [52]. Various factors, such as reproduction status, food availability, climatic conditions, and various stressors, might impact a host's immune response to virus replication [53]. An increase in rodent male testosterone levels in the spring is associated with humoral immunity inhibition. An experimental study found that injecting testosterone into male red voles can activate the latent TBE infection [54]. Furthermore, TBEV activation was seen in experimentally infected Syrian hamsters given vincristine, prednisolone, and adrenalin [55].

Vertical TBEV-Eu transmission in wild rodents was demonstrated in our study. TBEV RNA was detected in fetuses of *A. flavicollis*, *C. glareolus,* and *M. arvalis* females. To the best of our knowledge, no previous studies have found TBEV RNA in the fetuses of the aforementioned rodent species. However, vertical TBEV-Sib transmission from infected

laboratory mice and *C. rutilus* females has been described previously [56,57]. Vertical virus transmission was also demonstrated in an experimental study where TBEV was detected in up to 90% of experimentally infected red vole (*C. rutilus*) progeny. Moreover, transplacental TBEV transfer to embryos was revealed in naturally infected red voles [56]. In addition, sexual TBEV transmission in laboratory mice, which are not adapted hosts, was reported between infected males and uninfected females [58].

In both brain and internal organ mix suspensions, TBEV RNA was significantly more often found in *M. arvalis* compared to *A. flavicollis* and *C. glareolus*. Contrarily, TBEV RNA prevalence was significantly lower in suspension samples of *A. flavicollis* compared to other rodent species. However, results of virus isolation in cell culture demonstrated that the viral genome was absent in only five rodent specimens of *A. flavicollis* species, thus giving a 100% prevalence rate in other collected species. It is important to note that in our study, the majority (77.3%) of *M. arvalis* rodents were collected in January and March of 2020 when the average air temperature was 2.8–3.3 °C and ticks were not active. Consequently, it remains unclear if rodent species or the season of rodent collection was the influential factor for higher prevalence in both brain and internal organ mix suspension samples of *M. arvalis* before cultivation in cell culture.

A study conducted in the Czech Republic revealed that the abundance of *M. arvalis* voles was a significant factor explaining annual morbidity from TBEV and two other zoonoses [59]. TBEV RNA was detected in 11% of *M. arvalis* rodents collected in a non-risk area in Germany [15] and 6.2% in a high-risk area in Hungary [24]. Moreover, persistent TBEV infection for up to 100 dpi was reported in an experimentally infected *M. arvalis* in a study conducted by Achazi et al. [15]. However, *M. arvalis* voles are not a common rodent species in TBEV studies mainly because their main type of habitat is grasslands and not forests and woodlands, as it is for *I. ricinus* ticks and most common rodents in TBEV studies—*A. flavicollis* and *C. glareolus* [60]. Knap et al. [16] have suggested that *A. flavicollis* rodents might be less susceptible to TBEV infection compared to *C. glareolus*, although it has been implied that the rate of infection in a specific species might depend on the year. Moreover, it was suggested that the virus tropism of TBEV in *Apodemus* mice might be different from that in *Clethrionomys* rodents since the virus was predominantly detected in the spleen and less often in the brain, lungs, and blood clots of *Apodemus* rodents. Furthermore, the reported viral loads in the internal organs of *Apodemus* mice were generally lower compared to *Clethrionomys* voles [16]. In experimental studies, *C. glareolus* was demonstrated to produce higher viremia and higher antibody titers in comparison to the rodents of the *Apodemus* species [61,62]. However, regardless of the significant differences in the prevalence of viral RNA in suspension samples of *M. arvalis* and *A. flavicollis* compared to other species, results after sample cultivation in cell culture suggest that all rodent species included in the present study in TBEV foci were persistently infected with TBEV. This finding is in accordance with the study conducted by Achazi et al. [15], who also did not find a significant viral genome prevalence rate difference between rodent species.

There are a few limitations of our study that need to be addressed. The rodent sample size at different collecting sites and months differed, as did the number of rodents sampled for each species. Therefore, the interpretation of the results might not be completely accurate. Trapping of rodents was unevenly successful in different months and in different locations.

5. Conclusions

The present study revealed that in TBEV foci in Lithuania, the majority of wild rodents carry the TBE virus. The obtained results demonstrate that sample cultivation in cell culture is a highly efficient method of increasing viral load to detectable quantities. Moreover, the results suggest a higher chance of detecting TBEV RNA in rodents captured in lower average air temperatures than those suitable for ticks, which might help to reduce the number of rodents that have to be captured and analyzed to detect the virus in suspected TBEV foci.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v16030444/s1, Figure S1: Phylogenetic tree of the obtained TBEV isolate sequences based on NCR genome fragment; Table S1: Results of TBEV RNA prevalence rate in rodent brain and internal organ mix sample isolates which were PCR-negative in suspensions (n = 30), grouped according to the month of rodent trapping; Table S2: Results of TBEV RNA prevalence rate in brain and internal organ mix suspension and isolate samples of different rodent species.

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References

1. Růžek, D.; Dobler, G.; Mantke, O.D. Tick-borne encephalitis: Pathogenesis and clinical implications. *Travel Med. Infect. Dis.* **2010**, *8*, 223–232. [CrossRef] [PubMed]
2. European Centre for Disease Prevention and Control. Tick-Borne Encephalitis. Annual Epidemiological Report for 2020. Stockhom: ECDC. Available online: https://www.ecdc.europa.eu/en/publications-data/tick-borne-encephalitis-annual-epidemiological-report-2020 (accessed on 27 November 2023).
3. Korenberg, E.I. Natural focality of infections: Current problems and prospects of research. *Biol. Bull.* **2010**, *37*, 665–676. [CrossRef]
4. Mansfield, K.L.; Johnson, N.; Phipps, L.P.; Stephenson, J.R.; Fooks, A.R.; Solomon, T. Tick-borne encephalitis virus—A review of an emerging zoonosis. *J. Gen. Virol.* **2009**, *90*, 1781–1794. [CrossRef] [PubMed]
5. Borde, J.P.; Kaier, K.; Hehn, P.; Matzarakis, A.; Frey, S.; Bestehorn, M.; Dobler, G.; Chitimia-Dobler, L. The complex interplay of climate, TBEV vector dynamics and TBEV infection rates in ticks—Monitoring a natural TBEV focus in Germany, 2009–2018. *PLoS ONE* **2021**, *16*, e0244668. [CrossRef]
6. Randolph, S.E.; Green, R.M.; Peacey, M.F.; Rogers, D.J. Seasonal synchrony: The key to tick-borne encephalitis foci identified by satellite data. *Parasitology* **2000**, *121*, 15–23. [CrossRef]
7. Saegerman, C.; Humblet, M.F.; Leandri, M.; Gonzalez, G.; Heyman, P.; Sprong, H.; L'Hostis, M.; Moutailler, S.; Bonnet, S.I.; Haddad, N.; et al. First Expert Elicitation of Knowledge on Possible Drivers of Observed Increasing Human Cases of Tick-Borne Encephalitis in Europe. *Viruses* **2023**, *15*, 791. [CrossRef]
8. Daniel, M.; Danielová, V.; Fialová, A.; Malý, M.; Kříž, B.; Nuttall, P.A. Increased Relative Risk of Tick-Borne Encephalitis in Warmer Weather. *Front. Cell. Infect. Microbiol.* **2018**, *8*, 90. [CrossRef]
9. Randolph, S.E.; Asokliene, L.; Avsic-Zupanc, T.; Bormane, A.; Burri, C.; Gern, L.; Golovljova, I.; Hubalek, Z.; Knap, N.; Kondrusik, M.; et al. Variable spikes in tick-borne encephalitis incidence in 2006 independent of variable tick abundance but related to weather. *Parasit. Vectors* **2008**, *1*, 44. [CrossRef]
10. Jaenson, T.G.; Hjertqvist, M.; Bergström, T.; Lundkvist, Å. Why is tick-borne encephalitis increasing? A review of the key factors causing the increasing incidence of human TBE in Sweden. *Parasit. Vectors* **2012**, *5*, 184. [CrossRef]
11. Stefanoff, P.; Pfeffer, M.; Hellenbrand, W.; Rogalska, J.; Rühe, F.; Makówka, A.; Michalik, J.; Wodecka, B.; Rymaszewska, A.; Kiewra, D.; et al. Virus Detection in Questing Ticks is not a Sensitive Indicator for Risk Assessment of Tick-Borne Encephalitis in Humans: TBEV Detection in Ticks to Assess TBE Risk. *Zoonoses Public Health* **2013**, *60*, 215–226. [CrossRef]
12. Imhoff, M.; Hagedorn, P.; Schulze, Y.; Hellenbrand, W.; Pfeffer, M.; Niedrig, M. Review: Sentinels of tick-borne encephalitis risk. *Ticks Tick-Borne Dis.* **2015**, *6*, 592–600. [CrossRef]

13. Michelitsch, A.; Wernike, K.; Klaus, C.; Dobler, G.; Beer, M. Exploring the Reservoir Hosts of Tick-Borne Encephalitis Virus. *Viruses* **2019**, *11*, 669. [CrossRef]
14. Mikryukova, T.P.; Moskvitina, N.S.; Kononova, Y.V.; Korobitsyn, I.G.; Kartashov, M.Y.; Tyuten′kov, O.Y.; Protopopova, E.V.; Romanenko, V.N.; Chausov, E.V.; Gashkov, S.I.; et al. Surveillance of tick-borne encephalitis virus in wild birds and ticks in Tomsk city and its suburbs (Western Siberia). *Ticks Tick-Borne Dis.* **2014**, *5*, 145–151. [CrossRef]
15. Achazi, K.; Růžek, D.; Donoso-Mantke, O.; Schlegel, M.; Ali, H.S.; Wenk, M.; Schmidt-Chanasit, J.; Ohlmeyer, L.; Rühe, F.; Vor, T.; et al. Rodents as Sentinels for the Prevalence of Tick-Borne Encephalitis Virus. *Vector-Borne Zoonotic Dis.* **2011**, *11*, 641–647. [CrossRef]
16. Knap, N.; Korva, M.; Dolinšek, V.; Sekirnik, M.; Trilar, T.; Avšič-Županc, T. Patterns of Tick-Borne Encephalitis Virus Infection in Rodents in Slovenia. *Vector-Borne Zoonotic Dis.* **2012**, *12*, 236–242. [CrossRef]
17. Bakhvalova, V.N.; Dobrotvorsky, A.K.; Panov, V.V.; Matveeva, V.A.; Tkachev, S.E.; Morozova, O.V. Natural Tick-Borne Encephalitis Virus Infection among Wild Small Mammals in the Southeastern Part of Western Siberia, Russia. *Vector-Borne Zoonotic Dis.* **2006**, *6*, 32–41. [CrossRef]
18. Tonteri, E.; Jääskeläinen, A.E.; Tikkakoski, T.; Voutilainen, L.; Niemimaa, J.; Henttonen, H.; Vaheri, A.; Vapalahti, O. Tick-borne Encephalitis Virus in Wild Rodents in Winter, Finland, 2008–2009. *Emerg. Infect. Dis.* **2011**, *17*, 72–75. [CrossRef] [PubMed]
19. Süss, J. Epidemiology and ecology of TBE relevant to the production of effective vaccines. *Vaccine* **2003**, *21*, S19–S35. [CrossRef] [PubMed]
20. Sidorenko, M.; Radzijevskaja, J.; Mickevičius, S.; Bratčikovienė, N.; Paulauskas, A. Prevalence of Tick-borne encephalitis virus in questing *Dermacentor reticulatus* and *Ixodes ricinus* ticks in Lithuania. *Ticks Tick-Borne Dis.* **2021**, *12*, 101594. [CrossRef] [PubMed]
21. Schwaiger, M.; Cassinotti, P. Development of a quantitative real-time RT-PCR assay with internal control for the laboratory detection of tick borne encephalitis virus (TBEV) RNA. *J. Clin. Virol.* **2003**, *27*, 136–145. [CrossRef] [PubMed]
22. Puchhammer-Stöckl, E.; Kunz, C.; Mandl, C.W.; Heinz, F.X. Identification of tick-borne encephalitis virus ribonucleic acid in tick suspensions and in clinical specimens by a reverse transcription-nested polymerase chain reaction assay. *Clin. Diagn. Virol.* **1995**, *4*, 321–326. [CrossRef]
23. Grigas, J.; Simkute, E.; Simanavicius, M.; Pautienius, A.; Streimikyte-Mockeliune, Z.; Razukevicius, D.; Stankevicius, A. Hepatitis E genotype 3 virus isolate from wild boar is capable of replication in non-human primate and swine kidney cells and mouse neuroblastoma cells. *BMC Vet. Res.* **2020**, *16*, 95. [CrossRef]
24. Pintér, R.; Madai, M.; Horváth, G.; Németh, V.; Oldal, M.; Kemenesi, G.; Dallos, B.; Bányai, K.; Jakab, F. Molecular Detection and Phylogenetic Analysis of Tick-Borne Encephalitis Virus in Rodents Captured in the Transdanubian Region of Hungary. *Vector-Borne Zoonotic Dis.* **2014**, *14*, 621–624. [CrossRef]
25. Bakhvalova, V.N.; Chicherina, G.S.; Potapova, O.F.; Panov, V.V.; Glupov, V.V.; Potapov, M.A.; Seligman, S.J.; Morozova, O.V. Tick-Borne Encephalitis Virus Diversity in Ixodid Ticks and Small Mammals in South-Western Siberia, Russia. *Vector-Borne Zoonotic Dis.* **2016**, *16*, 541–549. [CrossRef]
26. Borde, J.P.; Glaser, R.; Braun, K.; Riach, N.; Hologa, R.; Kaier, K.; Chitimia-Dobler, L.; Dobler, G. Decoding the Geography of Natural TBEV Microfoci in Germany: A Geostatistical Approach Based on Land-Use Patterns and Climatological Conditions. *Int. J. Environ. Res. Public Health* **2022**, *19*, 11830. [CrossRef]
27. Žygutienė, M.; Alekseev, A.; Dubinina, H.; Kazlauskienė, R. Evidence for a risk of tick-borne infection in the city parks of Vilnius, Lithuania. *Ekologija* **2008**, *54*, 40–43. [CrossRef]
28. Radzišauskienė, D.; Žagminas, K.; Ašoklienė, L.; Jasionis, A.; Mameniškienė, R.; Ambrozaitis, A.; Jančorienė, L.; Jatužis, D.; Petraitytė, I.; Mockienė, E. Epidemiological patterns of tick-borne encephalitis in Lithuania and clinical features in adults in the light of the high incidence in recent years: A retrospective study. *Eur. J. Neurol.* **2018**, *25*, 268–274. [CrossRef] [PubMed]
29. Kiffner, C.; Vor, T.; Hagedorn, P.; Niedrig, M.; Rühe, F. Determinants of tick-borne encephalitis virus antibody presence in roe deer (*Capreolus capreolus*) sera. *Med. Vet. Entomol.* **2012**, *26*, 18–25. [CrossRef] [PubMed]
30. Jaenson, T.G.T.; Petersson, E.H.; Jaenson, D.G.E.; Kindberg, J.; Pettersson, J.H.O.; Hjertqvist, M.; Medlock, J.M.; Bengtsson, H. The importance of wildlife in the ecology and epidemiology of the TBE virus in Sweden: Incidence of human TBE correlates with abundance of deer and hares. *Parasit. Vectors.* **2018**, *11*, 477. [CrossRef]
31. Balčiauskas, L.; Kawata, Y. Red Deer in Lithuania: History, Status and Management. *Sustainability* **2022**, *14*, 14091. [CrossRef]
32. Katargina, O.; Russakova, S.; Geller, J.; Kondrusik, M.; Zajkowska, J.; Zygutiene, M.; Bormane, A.; Trofimova, J.; Golovljova, I. Detection and Characterization of Tick-Borne Encephalitis Virus in Baltic Countries and Eastern Poland. *PLoS ONE* **2013**, *8*, e61374. [CrossRef]
33. Han, X.; Juceviciene, A.; Uzcategui, N.Y.; Brummer-Korvenkontio, H.; Zygutiene, M.; Jääskeläinen, A.; Leinikki, P.; Vapalahti, O. Molecular epidemiology of tick-borne encephalitis virus in Ixodes ricinus ticks in Lithuania. *J. Med. Virol.* **2005**, *77*, 249–256. [CrossRef]
34. Pautienius, A.; Dudas, G.; Simkute, E.; Grigas, J.; Zakiene, I.; Paulauskas, A.; Armonaite, A.; Zienius, D.; Slyzius, E.; Stankevicius, A. Bulk Milk Tank Samples Are Suitable to Assess Circulation of Tick-Borne Encephalitis Virus in High Endemic Areas. *Viruses* **2021**, *13*, 1772. [CrossRef]
35. Pautienius, A.; Armonaite, A.; Simkute, E.; Zagrabskaite, R.; Buitkuviene, J.; Alpizar-Jara, R.; Grigas, J.; Zakiene, I.; Zienius, D.; Salomskas, A.; et al. Cross-Sectional Study on the Prevalence and Factors Influencing Occurrence of Tick-Borne Encephalitis in Horses in Lithuania. *Pathogens* **2021**, *10*, 140. [CrossRef]

36. Simkute, E.; Pautienius, A.; Grigas, J.; Urbute, P.; Stankevicius, A. The Prevalence, Seroprevalence, and Risk Factors of Tick-Borne Encephalitis Virus in Dogs in Lithuania, a Highly Endemic State. *Viruses* **2023**, *15*, 2265. [CrossRef]
37. Kozuch, O.; Gresíková, M.; Nosek, J.; Lichard, M.; Sekeyová, M. The role of small rodents and hedgehogs in a natural focus of tick-borne encephalitis. *Bull. World Health Organ.* **1967**, *36*, 61–66. [PubMed]
38. Růžek, D.; Vancová, M.; Tesařová, M.; Ahantarig, A.; Kopecký, J.; Grubhoffer, L. Morphological changes in human neural cells following tick-borne encephalitis virus infection. *J. Gen. Virol.* **2009**, *90*, 1649–1658. [CrossRef]
39. Ponomareva, E.P.; Ternovoi, V.A.; Mikryukova, T.P.; Protopopova, E.V.; Gladysheva, A.V.; Shvalov, A.N.; Konovalova, S.N.; Chausov, E.V.; Loktev, V.B. Adaptation of tick-borne encephalitis virus from human brain to different cell cultures induces multiple genomic substitutions. *Arch. Virol.* **2017**, *162*, 3151–3156. [CrossRef] [PubMed]
40. Morozova, O.V.; Panov, V.V.; Bakhvalova, V.N. Innate and adaptive immunity in wild rodents spontaneously and experimentally infected with the tick-borne encephalitis virus. *Infect. Genet. Evol.* **2020**, *80*, 104187. [CrossRef] [PubMed]
41. Michelitsch, A.; Fast, C.; Sick, F.; Tews, B.A.; Stiasny, K.; Bestehorn-Willmann, M.; Dobler, G.; Beer, M.; Wernike, K. Long-term presence of tick-borne encephalitis virus in experimentally infected bank voles (Myodes glareolus). *Ticks Tick-Borne Dis.* **2021**, *12*, 101693. [CrossRef] [PubMed]
42. Bakhvalova, V.N.; Panov, V.V.; Potapova, O.F.; Matveeva, L.E.; Morozova, O.V. Persistence of tick-borne encephalitis virus in organisms of wild small mammals and in permissive tissue cultures. *Far East. J. Infect. Pathol.* **2007**, *11*, 79–86. (In Russian)
43. Donoso Mantke, O.; Aberle, S.W.; Avšič-Županc, T.; Labuda, M.; Niedrig, M. Quality control assessment for the PCR diagnosis of tick-borne encephalitis virus infections. *J. Clin. Virol.* **2007**, *38*, 73–77. [CrossRef]
44. Nosek, J.; Grulich, I. The relationship between the tick-borne encephalitis virus and the ticks and mammals of the Tribec mountain range. *Bull. World Health Organ.* **1967**, *36*, 31–47.
45. Voyiatzaki, C.; Papailia, S.I.; Venetikou, M.S.; Pouris, J.; Tsoumani, M.E.; Papageorgiou, E.G. Climate Changes Exacerbate the Spread of Ixodes ricinus and the Occurrence of Lyme Borreliosis and Tick-Borne Encephalitis in Europe—How Climate Models Are Used as a Risk Assessment Approach for Tick-Borne Diseases. *Int. J. Environ. Res. Public Health* **2022**, *19*, 6516. [CrossRef]
46. Hudáčková, V.; Pekarčíková, J.; Peťko, B.; Mikulová, K.; Sivčo, P.; Rusňák, M. The impact of climatic conditions on the dynamics of tick-borne encephalitis in Slovakia in 2012–2016. *Epidemiol. Mikrobiol. Imunol.* **2023**, *72*, 78–85.
47. Korenberg, E.I.; YuV, K. Variation in parameters affecting risk of human disease due to TBE virus. *Folia Parasitol.* **1995**, *42*, 307–312.
48. Elväng, A.; Melik, W.; Bertrand, Y.; Lönn, M.; Johansson, M. Sequencing of a Tick-Borne Encephalitis Virus from *Ixodes ricinus* Reveals a Thermosensitive RNA Switch Significant for Virus Propagation in Ectothermic Arthropods. *Vector-Borne Zoonotic Dis.* **2011**, *11*, 649–658. [CrossRef]
49. Brandenburg, P.J.; Obiegala, A.; Schmuck, H.M.; Dobler, G.; Chitimia-Dobler, L.; Pfeffer, M. Seroprevalence of Tick-Borne Encephalitis (TBE) Virus Antibodies in Wild Rodents from Two Natural TBE Foci in Bavaria, Germany. *Pathogens* **2023**, *12*, 185. [CrossRef] [PubMed]
50. Moshkin, M.; Gerlinskaya, L.; Morozova, O.; Bakhvalova, V.; Evsikov, V. Behaviour, chemosignals and endocrine functions in male mice infected with tick-borne encephalitis virus. *Psychoneuroendocrinology* **2002**, *27*, 603–608. [CrossRef] [PubMed]
51. Bournez, L.; Umhang, G.; Moinet, M.; Richomme, C.; Demerson, J.M.; Caillot, C.; Legras, L.; Devillers, E.; Hansmann, Y.; Velay, A.; et al. Tick-Borne Encephalitis Virus: Seasonal and Annual Variation of Epidemiological Parameters Related to Nymph-to-Larva Transmission and Exposure of Small Mammals. *Pathogens* **2020**, *9*, 518. [CrossRef] [PubMed]
52. Moshkin, M.P.; Novikov, E.A.; Tkachev, S.E.; Vlasov, V.V. Epidemiology of a tick-borne viral infection: Theoretical insights and practical implications for public health. *BioEssays* **2009**, *31*, 620–628. [CrossRef] [PubMed]
53. Viney, M.; Riley, E.M. The Immunology of Wild Rodents: Current Status and Future Prospects. *Front. Immunol.* **2017**, *8*, 1481. [CrossRef] [PubMed]
54. Bakhvalova, V.N.; Dobrotvorsky, A.K.; Morozova, O.V.; Matveeva, V.A.; Tkachev, S.E.; et al. Persistence and activation of tick-borne encephalitis virus in small mammals in forest-steep zone of South Siberia. In *Modern Situation and Perspectives of Combat with Tick-Borne Infections in XXI Century*; Tomsk State University (TSU): Tomsk, Russia, 2006; pp. 19–21. (In Russian)
55. Frolova, T.V.; Pogodina, V.V. Activating effect of adrenaline, prednisolone and vincristine in the late periods of tick-borne encephalitis virus persistence. *Vopr. Virusol.* **1984**, *29*, 103–108. (In Russian) [PubMed]
56. Bakhvalova, V.N.; Potapova, O.F.; Panov, V.V.; Morozova, O.V. Vertical transmission of tick-borne encephalitis virus between generations of adapted reservoir small rodents. *Virus Res.* **2009**, *140*, 172–178. [CrossRef] [PubMed]
57. Molnárová, A.; Mayer, V. Experimental infection of pregnant mice with viruses of the tick-borne encephalitis (TBE) complex. *Acta Virol.* **1980**, *24*, 297.
58. Gerlinskaia, L.A.; Bakhvalova, V.N.; Morozova, O.V.; Tsekhanovskaia, N.A.; Matveeva, V.A.; Moshkin, M.P. Sexual transmission of tick-borne encephalitis virus in laboratory mice. *Bull. Exp. Biol. Med.* **1997**, *123*, 327–328. (In Russian) [CrossRef]
59. Hubálek, Z. Effect of environmental variables on incidence of tick-borne encephalitis, leptospirosis and tularemia. *Cent. Eur. J. Public Health* **2021**, *29*, 187–190. [CrossRef]
60. Król, N.; Obiegala, A.; Imholt, C.; Arz, C.; Schmidt, E.; Jeske, K.; Ulrich, R.G.; Rentería-Solís, Z.; Jacob, J.; Pfeffer, M. Diversity of *Borrelia burgdorferi* sensu lato in ticks and small mammals from different habitats. *Parasites Vectors* **2022**, *15*, 195. [CrossRef]

61. Heigl, Z.; von Zeipel, G. Experimental Infection with Tick-Borne Encephalitis Virus in Clethrionomys glareolus, Apodemus flavicollis, Apodemus sylvaticus and Mus musculus. I. Virological Studies. *Acta Pathol. Microbiol. Scand.* **1966**, *66*, 489–509. [CrossRef]
62. Von Zeipel, G.; Heigl, Z. Experimental infection with tick-borne virus in Clethrionomys glareolus, Apodemus flavicollis, Apodemus sylvaticus and Mus musculus. 2. Serological studies. *Acta Pathol. Microbiol. Scand.* **1966**, *68*, 211–229. [CrossRef]

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SUPPLEMENTARY MATERIAL

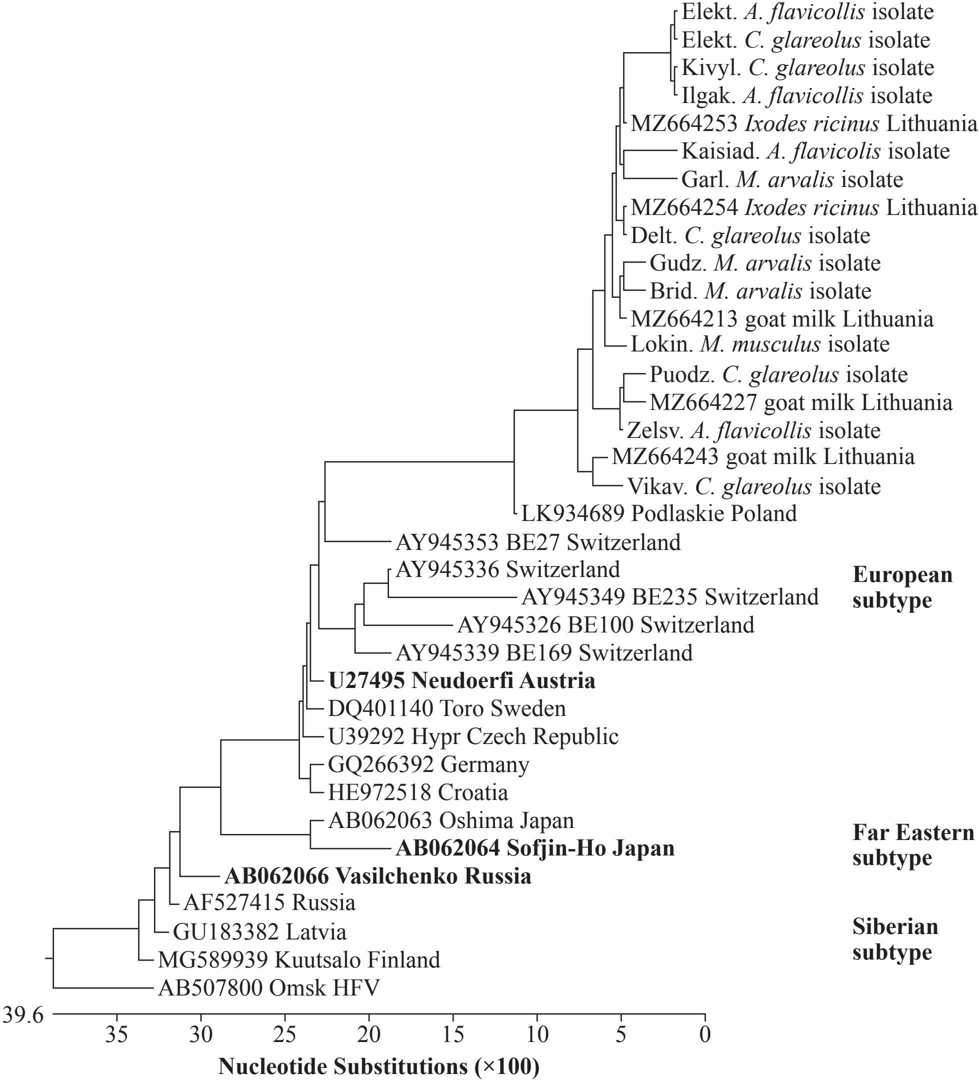

Fig. S1. *Phylogenetic tree of the obtained TBEV isolate sequences based on NCR genome fragment. The Omsk hemorrhagic fever virus was used as an outgroup. Sequences of different TBEV strains and closely related flaviviruses chosen from the National Center for Biotechnology Information (NCBI) GenBank database were used for phylogenetic comparisons. Multiple alignment of all sequences was created using ClustalW software (Clustal, Dublin, Ireland) in the MEGA X package. The neighbor-joining method was used for phylogenetic tree construction with 100 bootstrapping replicates*

Table S1. *Results of TBEV RNA prevalence rate in rodent brain and internal organ mix sample isolates that were PCR-negative in suspensions (n = 30), grouped according to the month of rodent trapping*

Date of rodent trapping	**Number of trapped rodents (total/TBEV RNA-positive in suspensions/ TBEV RNA-positive in isolates)**	**TBEV RNA prevalence rate (%) in suspension samples**	**TBEV RNA prevalence rate (%) in rodent sample isolates which were PCR-negative in suspensions**	**Average monthly air tempe-rature (°C)**	**Rodent trapping sites and TBEV RNA prevalence rate (%) in suspension samples**
March 2019	34/28/33	82.4	15.2 (95% CI 5.1–31.9)[1]	3 (1–3.7)	Elektrenai (84.6); Garliava (72.7); Grabuciskes (100); Ilgakiemis (90)
April 2019	8/5/8	62.5	37.5 (95% CI 8.5–75.5)[2]	8.6 (5–15.1)	Bridai (25); Narepai (100)
October 2019	24/15/22	62.5	31.8 (95% CI 13.9–54.9)	9.3 (6–13.2)	Kaisiadorys (42.9); Naujasodis (60); Pakalniskes (50); Zelsva (87.5)
November 2019	23/15/21	65.2	28.6 (95% CI 11.3–52.2)	4.9 (–1.2–8.3)	Kazlu Ruda (62.5); Krokialaukis (66.7)
January 2020	31/28/31	90.3	9.7 (95% CI 2.0–25.8)[2]	2.8 (0.6–5.1)	Gudziunai (100); Kivyliai (100); Puodziai (70); Elektrenai (100)
March 2020	9/9/9	100	0 (95% CI 0–33.6)*	3.3 (0.5–5.5)	Deltuva (100); Lokine (100)
May 2020	10/4/10	40	60 (95% CI 26.2–87.8)	10.2 (7.2–13.1)	Lekeciai (25); Vilkaviskis (50)

Note: [1] Including rodents that were PCR-positive only in fetal sample suspension. [2] Including rodents whose brain sample was not taken. *One-sided 97.5% confidence interval.

Table S2. *Results of TBEV RNA prevalence rate in brain and internal organ mix suspension and isolate samples of different rodent species*

Name of the rodent trapping location	**Lati-tude coordi-nate**	**Longi-tude coordi-nate**	**Date of rodent trapping**	**Number and species of trapped rodents (total/TBEV RNA positive in suspen-sions/TBEV RNA positive in isolates)**	**TBEV RNA prevalence rate in suspension samples (%)**	**TBEV RNA prevalence rate in isolate samples (%)**
Gudziunai	56.0888	23.2805	January 2020	*A. flavicollis* 2/2/2 *C. glareolus* 1/1/1 *M. arvalis* 9/9/9	100	100
Bridai	56.0226	23.3263	April 2019	*A. flavicollis* 2/0/2 *M. arvalis* 2/1/2	25	100
Deltuva	55.2857	24.6144	March 2020	*C. glaroelus* 1/1/1 *M. musculus* 1/1/1	100	100
Elektrenai[1]	54.7763	24.6290	March 2019	*A. flavicollis* 8/6/8 *C. glareolus* 1/1/1 *M. arvalis* 1/1/1	84.6	100
			January 2020	*A. flavicollis* 2/2/2 *C. glareolus* 1/1/1	100	100
Garliava	54.8011	23.8756	March 2019	*A. flavicollis* 8/7/8* *C. glareolus* 2/1/2 *M. arvalis* 1/1/1	72.7	100
Grabuciske s	54.8947	24.1574	March 2019	*M. arvalis* 2/2/2	100	100
Ilgakiemis	54.7860	23.8677	March 2019	*A. flavicollis* 4/3/3 *C. glareolus* 2/2/2 *M. arvalis* 3/3/3 *M. musculus* 1/1/1	90	90
Kaisiadorys	54.8511	24.4561	October 2019	*A. flavicollis* 5/2/3 *M. musculus* 1/0/1 *A. agrarius* 1/1/1	42.9	71.4
Kazlu Ruda	54.7371	23.4609	November 2019	*A. flavicollis* 5/4/5 *C. glareolus* 3/1/3	62.5	100
Kivyliai	56.3566	22.7115	January 2020	*C. glareolus* 5/5/5 *M. arvalis* 1/1/1	100	100
Krokialauki s	54.4277	23.4847	November 2019	*A. flavicollis* 12/7/10 *C. glareolus* 1/1/1 *A. sylvaticus* 2/2/2	66.7	86.7
Lekeciai	54.5213	23.0375	May 2020	*A. flavicollis* 2/0/2 *M. musculus* 2/1/2	25	100
Lokine	54.3275	24.4900	March 2020	*A. flavicollis* 4/4/4 *M. musculus* 3/3/3	100	100
Narepai	54.9491	24.0171	April 2019	*A. flavicollis* 1/1/1 *C. glareolus* 3/3/3*	100	100
Naujasodis	54.9517	23.9873	October 2019	*A. flavicollis* 5/3/5	60	100

Table S2. Continued

Name of the rodent trapping location	Latitude coordinate	Longitude coordinate	Date of rodent trapping	Number and species of trapped rodents (total/TBEV RNA positive in suspensions/TBEV RNA positive in isolates)	TBEV RNA prevalence rate in suspension samples (%)	TBEV RNA prevalence rate in isolate samples (%)
Pakalniskes	54.7371	24.6999	October 2019	*A. flavicollis* 2/0/2 *C. glareolus* 1/1/1 *M. arvalis* 1/1/1	50	100
Puodziai	54.3580	24.6600	January 2020	*A. flavicollis* 4/4/4 *C. glareolus* 6/3/6	70	100
Vilkaviskis	54.6294	23.1872	May 2020	*A. flavicollis* 1/0/1* *C. glareolus* 2/0/2* *M. arvalis* 2/2/2* *M. musculus* 1/1/1	50	100
Zelsva	54.4070	23.4578	October 2019	*A. flavicollis* 8/7/8	87.5	100

Note: [1] Samples of rodents were collected at different months in the same location. [*] A fetal sample was taken from rodents.

Table S3. *Data of PCR-positive dogs*

No.	Data of the breed, age, sex, anamnesis, outcome of the TBE disease and status of TBEV-specific antibodies in blood serum of TBEV RNA PCR-positive dogs
1.	At 25/09/2021 Mixed breed female dog of 3 months of age was presented to the veterinary clinic with a history of vomiting. The owners got the dog few days ago, administered anthelmintic drug at home, after which the dog started to vomit. The dog was presented to the clinic non-ambulatory. Hypothermia (37 °C) and hypotension were observed. Moderate juvenile anemia with mild regeneration, severe leucopenia, severe toxic changes in cytoplasm of neutrophils were observed on blood smear. Parvo/Corona/Giardia antigen test was negative. The dog showed improvement two days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
2.	*At 09/10/2020 German Shepherd breed male dog of 5 months of age was presented to veterinary clinic with a history of babesiosis infection two weeks prior. The dog was non-ambulatory, impairment of facial reflexes and vision were observed along with episodes of seizures (10 times a day for about 30 seconds). Bradycardia, tachypnoea, lymphadenopathy of submandibular and popliteal lymph nodes was observed. Moderate reticulocytosis, mild anemia, severe toxic changes in cytoplasm of neutrophils, moderate eosinophilia with 14% of band eosinophils, moderately reactive monocytes, moderate thrombocytosis and moderate number of macrocytic platelets were observed on blood smear. On the following day, TBEV RNA was detected CSF. Seizures with “swimming movements” were observed. seizures. On the third day, coordination of the dog was impaired, all limbs were weak, the dog tended to rotate to the left side and showed rotational movements. Menace reflex was impaired. On the fifth day vision, walking and appetite improved. On the seventh day menace reflex showed improvement. Mild anemia, severe monocytosis, mild eosinophilia and reactive lymphocytes were observed on blood smear. Ten days after initial appointment respiration of the dog impaired. The dog showed depression, dullness, heavy breathing, bruxism, head tilt to the left and rotational movements twelve days after initial appointment. The dog showed improvement of clinical signs two weeks after therapy was initiated. However, the reduced paw replacement remained. ELISA test of TBEV-specific antibodies was negative.
3.	At 30/05/2020 Fox terrier breed female dog of 5 months of age was presented to veterinary clinic with a history of depression, dullness, inappetence, diarrhea and occasional vomiting from the past two days. Mild anemia and mild neutrophilia were observed on blood smear. The dog showed improvement three days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
4.	At 31/10/2020 Akita breed female dog of 6 months of age was presented at veterinary clinic with a history of a trauma after bumping his side of body when climbing stairs. The state of dog rapidly worsened. The dog showed diarrhea, vomiting, stiff walk, hyperthermia (39.5 °C) and hyperventilation. Regardless of intensive care and treatment dog died of hydrothorax. ELISA test of TBEV-specific antibodies was negative.
5.	At 02/10/2020 Siberian Husky breed male dog of 6 months of age was presented to veterinary with a history of depression and dullness from the past few days. The owners found an attached tick few days prior. Fever (39.8 °C) was observed. *Babesia* spp. in erythrocytes, moderate neutropenia, toxic changes in cytoplasm of neutrophils and moderate thrombocytopenia were observed on blood smear. The dog showed improvement two days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
6.	*At 05/11/2020 German Shepherd breed female dog of 10 months of age was presented to veterinary clinic with a history of depression, dullness and inappetence from the past few days. Attached tick was found on the body of the dog. Fever (40.7 °C) was observed. *Babesia* spp. in erythrocytes, mild anemia, mild toxic changes in cytoplasm of neutrophils, mild monocytosis, moderately reactive monocytes and moderate thrombocytopenia were observed on blood smear. The dog showed improvement two days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.

Table S3. *Continued*

No.	Data of the breed, age, sex, anamnesis, outcome of the TBE disease and status of TBEV-specific antibodies in blood serum of TBEV RNA PCR-positive dogs
7.	At 30/05/2020 Mixed breed female dog of 10 months of age was presented to veterinary clinic with a history of depression and inappetence from the past few days. Few attached ticks were found on the body of the dog about two weeks prior. Mild anemia with mild regeneration, highly reactive monocytes, moderately reactive lymphocytes and macrocytic platelets were observed on blood smear. On the third day, the dog was sleeping more than usual and intake of food was reduced. Mild anemia, highly reactive monocytes and moderate toxic changes in cytoplasm of neutrophils were observed on blood smear. PCR test for diagnosis of babesiosis performed at the clinic was negative. The dog showed improvement three days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
8.	At 10/11/2020 Lagotto Romagnollo breed male dog of 1 year of age was presented to veterinary clinic with a history of depression, reduced activity, weakness in hind limbs and difficulty standing up for the past few days. The dog showed improvement three days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
9.	At 19/11/2020 Shih Tzu breed male dog of 1 year of age was presented to veterinary clinic with a history of inability to jump up on the bed at night and vomiting in the morning of presentation at the clinic. Clinical examination revealed lack of coordination. Mild neutrophilia was observed on blood smear. The dog showed improvement three days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
10.	At 31/05/2020 Miniature Pinscher breed male dog of 1 year of age was presented to veterinary clinic with a history of difficulty standing up, reluctance to walk and stiffness of the body. Moderate reticulocytosis and highly reactive lymphocytes were observed on blood smear. The dog showed improvement three days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
11.	At 06/09/2021 Mixed breed male dog of 1 year of age was presented to veterinary clinic with a history of limping with front left limb for the past few days. Clinical examination revealed hypersensitivity of the elbow region. No external injuries were observed. Attached tick was found by the owner on the day before the visit to the clinic. Moderate toxic changes in the cytoplasm of neutrophils and mild eosinophilia were observed on blood smear. The dog showed improvement two days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
12.	At 16/10/2020 Mixed breed female dog of 1 year of age was presented to veterinary clinic for a prophylactic annual testing. ELISA test of TBEV-specific antibodies was negative.
13.	At 16/11/2020 German Shepherd breed female dog of 1 year of age was presented to veterinary clinic was presented with a history of depression, dullness and inappetence from the past few days. Hyperthermia (39.4 °C) was observed. Moderate neutrophilia, mild toxic changes in the cytoplasm of neutrophils, moderate monocytosis and reactive monocytes were observed on blood smear. CRP was > 225 mg/L. Blood culture for diagnosis of sepsis was negative. The dog showed improvement three days after therapy initiation. Moderate neutrophilia, 5% of band neutrophils, mild toxic changes in cytoplasm of neutrophils and moderate monocytosis were observed on blood smear three days following the initial appointment. ELISA test of TBEV-specific antibodies was negative.
14.	At 10/05/2021Golden Retriever breed female dog of 1 year of age was presented to veterinary clinic with a history of depression, dullness, inappetence, diarrhea and vomiting from the past few days. Seven attached ticks were found on the body of the dog. Hyperthermia (39.4 °C) was observed. Mild anemia, moderately reactive monocytes and mild thrombocytopenia were observed on blood smear. The dog showed improvement two days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.

Table S3. *Continued*

No.	Data of the breed, age, sex, anamnesis, outcome of the TBE disease and status of TBEV-specific antibodies in blood serum of TBEV RNA PCR-positive dogs
15.	At 15/05/2021Swiss Shepherd breed male dog of 1 year of age was presented to veterinary clinic with a history of depression, dullness, inappetence and diarrhea from the past few days. Few attached ticks were found by owners at home and two ticks were found at the clinic. Hyperthermia (39.8 °C) was observed. The dog showed improvement two days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
16.	At 09/10/2021 Doberman breed male dog of 1 year of age was presented to veterinary clinic with a history of depression, dullness and inappetence from the past few days. The dog had access to free roam in the woods. Mildly elevated number of eosinophils was observed on blood smear. Fecal cytology revealed dysbacteriosis, moderate amount of mucus and occasional neutrophils. The dog showed improvement three days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
17.	At 12/07/2020 Shiba-Inu breed male dog of 2 years of age was presented to veterinary clinic with a history of diarrhea, impaired walking and stiffness of the body from the past two days. Mild anemia and moderate neutrophilia were observed on blood smear. The dog showed improvement the day after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
18.	At 19/10/2021 Biewer Terrier breed male dog of 2 years of age was presented to veterinary clinic with a history of behavior changes, shivering and inappetence from the past two days. CRP was < 10 mg/L. Mild eosinophilia was observed on blood smear. The dog showed improvement of two days after therapy initiation. TBEV-specific antibodies of 73 NTU/mL were detected by ELISA.
19.	At 07/07/2020 Bernese Mountain Dog breed female dog of 2 years of age was presented to veterinary clinic with a history of depression and inappetence from the past two days. Anaplasmosis was diagnosed in another clinic using Caniv4 test a week prior. Specific *Anaplasma* spp. morulae in neutrophils, mild eosinophilia and severe thrombocytopenia were observed on blood smear. The dog showed improvement four days after therapy initiation. TBEV-specific antibodies of 207 NTU/mL were detected by ELISA.
20.	*At 18/11/2020 Akita breed female dog of 2 years of age was presented to veterinary clinic with a history of depression, dullness and inappetence from the past few days. Three attached ticks were found on the body of the dog. Hyperthermia (40.1 °C) was observed. Babesia spp. in erythrocytes, mild anemia, mild neutrophilia, mild toxic changes in cytoplasm of neutrophils, moderately reactive monocytes and moderate thrombocytopenia were observed on blood smear. The dog showed improvement two days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
21.	At 05/12/2020 Labrador Retriever breed female dog of 2 years of age was presented to veterinary clinic with a history of trauma during jumping and playing outside. Mass lesion was observed in the lungs in X-ray. The dog was euthanized. TBEV-specific antibodies of 344 NTU/mL were detected by ELISA.
22.	At 25/05/2020 Bichon Frise breed female dog of 2 years of age was presented to veterinary clinic with a history of depression and inappetence from the past few days. Attached tick was found on the body of the dog. The dog showed improvement two days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
23.	At 14/09/2021 West Scottish Terrier breed female dog of 2 years of age was presented to veterinary clinic with a history of inappetence, diarrhea and occasional vomiting from the past few days. Diet change was initiated a week prior. Fecal cytology revealed dysbacteriosis. The dog showed improvement one week after therapy initiation. TBEV-specific antibodies of 106 NTU/mL were detected by ELISA.

Table S3. *Continued*

No.	**Data of the breed, age, sex, anamnesis, outcome of the TBE disease and status of TBEV-specific antibodies in blood serum of TBEV RNA PCR-positive dogs**
24.	At 11/09/2021 German Shepherd breed male dog of 2 years of age was presented to veterinary clinic with a history of depression, dullness and inappetence from the past few days. Hyperthermia (40.7 °C) and hematuria were observed. Attached ticks were found multiple times in the past month. *Babesia* spp. in erythrocytes, moderate anemia, mild neutrophilia, moderate toxic changes in cytoplasm of neutrophils, moderate monocytosis, highly reactive monocytes and severe thrombocytopenia were observed on blood smear. The dog showed improvement two days after therapy initiation. TBEV-specific antibodies of 474 NTU/mL were detected by ELISA.
25.	At 21/10/2020 Akita breed male dog of 3 years of age was presented to veterinary clinic with a history of reduced activity and weakness in hind limbs from the past few days. The dog had babesiosis five months ago which resolved after treatment. The dog showed improvement two days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
26.	At 13/06/2020 Rottweiler breed male dog of 3 years of age was presented to veterinary clinic with a history of depression, dullness, lethargy, inappetence and vomiting. Hyperthermia (39.5 °C) was observed. Gastric dilatation-volvulus was diagnosed and the surgical treatment was applied immediately. The dog showed improvement after therapy initiation. TBEV-specific antibodies of 54 NTU/mL were detected by ELISA.
27.	At 15/10/2020 Swiss Shepherd breed male dog of 3 years of age was presented to veterinary clinic with a history of sensitive gastrointestinal tract and occasional vomiting in the past month. *Dirofilaria repens* were found on blood smear five days prior. One week after initial appointment, hemorrhagic gastritis was suspected following gastroscopy, although the histopathological examination did not show any changes in the gastric mucosa. Two weeks after initial appointment the dog was depressed, but did not vomit for a week. Moderate reticulocytosis, 1% of nucleated erythrocytes, mild neutrophilia, 7% of band neutrophils, mild monocytosis, mildly reactive monocytes and reactive lymphocytes were observed on blood smear. Blood transfusion was performed. The dog showed improvement after the blood transfusion. TBEV-specific antibodies of 41 NTU/mL were detected by ELISA.
28.	At 03/10/2021 Akita breed male dog of 3 years of age was presented to veterinary clinic with a history of *Babesia* spp. infection which was diagnosed and treated in another clinic. Hyperthermia (39.4 °C) was observed. Moderate number of spherocytes, severe leucopenia, reactive monocytes and severe thrombocytopenia were observed on blood smear. On the third day, severe monocytosis, highly reactive monocytes, 10% of band neutrophils and severe thrombocytopenia were observed on blood smear. The dog showed inappetence and hyperthermia (40.5 °C) four days after initial appointment. One month after the initial appointment, moderate anemia with mild regeneration, moderate number of spherocytes, moderate number of erythrocytes with Heinz bodies, moderate leukopenia, mild eosinophilia and severe thrombocytopenia were observed on blood smear. Splenectomy and blood transfusion were performed, followed by death two months after initial appointment. Histopathological examination of the spleen revealed extramedullary haematopoiesis with white pulp atrophy. TBEV-specific antibodies of 42 NTU/mL were detected by ELISA.
29.	At 01/10/2021 mixed breed male dog of 3 years of age was presented to veterinary clinic with a history of depression and inappetence from the past few days. Complications after osteosynthesis (which was performed 20 days ago to treat the fraction of tibia) had occurred and the osteosynthesis screw was dislocated into the joint and subcutis. Osteomyelitis was suspected. Moderate leukocytosis, monocytosis, reactive monocytes, moderate toxic changes in cytoplasm of neutrophils, 20% of band neutrophils and moderately reactive lymphocytes were observed on blood smear. The following day the dog showed depression, dullness, hyperthermia (40 °C) and occasional vomiting. CRP was 270 mg/L. The dog showed improvement six days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.

Table S3. Continued

No.	Data of the breed, age, sex, anamnesis, outcome of the TBE disease and status of TBEV-specific antibodies in blood serum of TBEV RNA PCR-positive dogs
30.	At 30/09/2021 Cesky Terrier breed female dog of 3 years of age was presented to veterinary clinic with a history of vomiting with blood. Hyperthermia (39.4 °C) was observed. Foreign body (fishing-line) was found in the stomach during gastroscopy and was surgically removed. Moderate leukocytosis, highly toxic changes in cytoplasm of neutrophils, 17% of band neutrophils and highly reactive monocytes were observed in blood smear. CRP was 215 mg/L. The dog showed improvement two days after surgical therapy initiation. ELISA test of TBEV-specific antibodies was negative.
31.	At 01/11/2021 mixed breed female dog of 3 years of age was presented to veterinary clinic with a history of depression, dullness and occasional vomiting from the past two days. Clinical examination of the dog revealed pale mucous membranes and mild abdominal breathing. Moderate regenerating anemia, moderate neutrophilia and severe thrombocytopenia were observed on blood smear. The dog was suspected to be intoxicated with rodenticide. Hydrothorax was observed in X-ray. Regardless of intensive care and treatment the dog died the next morning. ELISA test of TBEV-specific antibodies was negative.
32.	*At 23/10/2020 German Shepherd breed male dog of 4 years of age was presented to veterinary clinic with a history of occasional vomiting from the past two days. Hyperthermia (39.3 °C) was observed. The dog showed depression, dullness, inappetence, diarrhea and hyperthermia (39.6 °C) two days after the initial appointment. The dog showed improvement three days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
33.	At 05/11/2020 Scotland Terrier breed male dog of 4 years of age was presented to veterinary clinic with a history of small skin wounds and scabs on the body. The dog showed improvement one week after therapy initiation. TBEV-specific antibodies of 43 NTU/mL were detected by ELISA.
34.	At 25/04/2021 King Charles Spaniel breed male dog of 4 years of age was presented to veterinary clinic with a history depression, inappetence, vocalization, yelping, reluctance to walk and shivering from the past three days. Hyperthermia (39.4 °C) was observed. An attached tick was found on the body of the dog two weeks ago. The dog showed improvement two days after therapy initiation. TBEV-specific antibodies of 31 NTU/mL were detected by ELISA.
35.	At 25/20/2020 Border Collie breed female dog of 4 years of age was presented to veterinary clinic with a history of depression, dullness and anemic mucous membranes. *Babesia* spp. infection was diagnosed and treated in another clinic ten days prior. Moderate reticulocytosis, 1% of nucleated erythrocytes, severe neutrophilia, highly toxic changes in cytoplasm of neutrophils, 13% of band neutrophils, monocytosis with occasional reactive monocytes and mild thrombocytopenia with moderate number of macrocytic platelets were observed on blood smear. Blood serum was icteric, agglutination test was negative. The dog showed improvement two days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
36.	At 20/10/2020 Belgian Malinois breed male dog of 4 years of age was presented to veterinary clinic with a history of eating leaves and possibly a small branch of a tree. Frequent vomiting (about ten times) with bile and blood were observed. Mild neutrophilia, 7% of band neutrophils, mild monocytosis and occasional reactive monocytes were observed on blood smear. The dog showed improvement the following day after therapy initiation. TBEV-specific antibodies of 525 NTU/mL were detected by ELISA.
37.	At 16/09/2021 Chihuahua breed male dog of 4 years of age was presented to veterinary clinic with a history of diarrhea and occasional vomiting from the past two days. Bones were fed to the dog three days prior. Mild leukocytosis was observed on blood smear. CRP was 71.5 mg/L. Mild leukocytosis, reactive lymphocytes and mild eosinophilia were observed on blood smear two days after initial appointment. The dog showed improvement three days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.

Table S3. *Continued*

No.	**Data of the breed, age, sex, anamnesis, outcome of the TBE disease and status of TBEV-specific antibodies in blood serum of TBEV RNA PCR-positive dogs**
38.	At 26/10/2021 Yorkshire Terrier breed male dog of 4 years of age was presented to veterinary clinic with a history of dullness and diarrhea with blood from the past two days. Mild neutrophilia, mild toxic changes in cytoplasm of neutrophils, mild monocytosis and occasional reactive lymphocytes were observed on blood smear. The dog showed improvement three days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
39.	At 21/10/ 2020 mixed breed male dog of 5 years of age was presented to veterinary clinic with a history of injuries after an encounter with another dog. The dog showed improvement on the following day after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
40.	At 01/09/2021 mixed breed male dog of 5 years of age was presented to veterinary clinic with a history of painful and enlarged caudal thoracic mammary gland from the past few days. Moderate periodontitis was observed. Mild anemia, mild neutrophilia, 10% of band neutrophils, mild toxic changes in cytoplasm of neutrophils, mild monocytosis and occasional reactive monocytes were observed on blood smear. CRP was 90.4 mg/L. The dog showed improvement two days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
41.	At 15/06/2020 Australian Shepherd breed male dog of 6 years of age was presented to veterinary clinic with a history of trauma after a chase in the woods. The dog was found by owner laying on his side and paralyzed. Clinical examination of the dog revealed head tilt to the left side and reduced response to paw replacement. Hyperthermia (39.7 °C) was observed. On the following day, tetraplegia was observed. Mild neutrophilia, moderate toxic changes in cytoplasm of neutrophils and mild thrombocytopenia were observed on blood smear. Impaired urination and defecation were observed three days after trauma. Facial nerve reflexes and reflexes of biceps, triceps and patella were normal. Sensitivity of limbs and ability to urinate recovered five days after trauma. MRT showed injury in C4-5. The dog was able to stand up and walk one week after trauma. However, the dog showed depression, dullness, inappetence, diarrhea and occasional vomiting. Neutrophilia, 6% of band neutrophils, moderate monocytosis with reactive monocytes and mild thrombocytopenia were observed on blood smear. Hyperventilation and panting were observed six days after trauma. Moderate neutrophilia, 19% of band neutrophils and specific *A. phagocytophilum* morulae in neutrophils and highly reactive monocytes were observed on blood smear. The dog was lethargic, showed hyperventilation and hydrothorax was diagnosed twenty days after trauma. Cytology of thoracic punctate revealed moderate number of neutrophils, macrophages, erythrocytes, and reactive lymphocytes with cytoplasmic granules. Moderate neutrophilia, 16% of band neutrophils, mild toxic changes in cytoplasm of neutrophils, occasional reactive lymphocytes, reactive monocytes and mild thrombocytopenia were observed on blood smear. Two days after diagnosis of hydrothorax, the dog showed improvement. Anemia was diagnosed three days after improvement and the therapy was continued for three weeks. TBEV-specific antibodies of 450 NTU/mL were detected by ELISA.
42.	At 08/10/2020 Akita breed male dog of 6 years of age was presented to veterinary clinic with a history of dullness, depression, inappetence, reluctance to walk and stiffness of the body from the past three days. Hyperthermia (40.1 °C) was observed. Mild anemia, mild neutrophilia and mild monocytosis were observed on blood smear. The dog showed improvement two days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.

Table S3. *Continued*

No.	Data of the breed, age, sex, anamnesis, outcome of the TBE disease and status of TBEV-specific antibodies in blood serum of TBEV RNA PCR-positive dogs
43.	*At 10/11/2020 German Shepherd breed male dog of 6 years of age was presented to veterinary clinic with a history of cataplexy-like symptoms. No seizures were observed. Two attached ticks were noticed by owners two weeks prior. Hyperthermia (39.7 °C) was observed. Babesia spp. in erythrocytes, microcytic non-regenerating anemia, moderate number of spherocytes, moderate neutropenia, highly reactive lymphocytes and moderate thrombocytopenia were observed on blood smear. The dog showed improvement on the following day after therapy initiation. Ten days after initial appointment, mild monocytosis, mild thrombocytopenia and macrocytic platelets were observed on blood smear. TBEV-specific antibodies of 217 NTU/mL were detected by ELISA.
44.	At 19/09/2021 German Hunting Terrier breed male dog of 6 years of age was presented to veterinary clinic with a history of wild boar attack and multiple wounds. Mild anemia, moderate neutrophilia, moderate toxic changes in cytoplasm of neutrophils, 10% of band neutrophils, moderate monocytosis and moderately reactive monocytes were observed on blood smear. The dog showed improvement three days after therapy initiation. TBEV-specific antibodies of 348 NTU/mL were detected by ELISA.
45.	At 19/09/2021 mixed breed female dog of 6 years of age was presented to veterinary clinic with a history of depression, dullness, inappetence and weight loss from the past week. Stress leukogram was observed on blood smear. The dog showed improvement four days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
46.	At 18/10/2020 German Shepherd breed male dog of 7 years of age was presented to veterinary clinic with a history of vomiting following an escape to roam free outside at night. Moderate number of spherocytes, severe neutrophilia, 14% of band neutrophils, occasional reactive monocytes and moderate thrombocytopenia were observed on blood smear. Cytology of CSF revealed erythrocytes and moderate number of neutrophils two days after initial appointment. The dog showed somnolence and vomiting. Seizures were observed and the dog was euthanized three days after initial appointment. ELISA test of TBEV-specific antibodies was negative.
47.	At 26/05/2021 mixed breed female dog of 7 years of age was presented to veterinary clinic with a history of depression, dullness, inappetence and occasional vomiting from the past few days. Hyperthermia (39.7 °C) was observed. The dog showed improvement two days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
48.	At 27/05/2020 Akita breed female dog of 7 years of age was presented to veterinary clinic with a history of depression, dullness and inappetence from the past few days. Hyperthermia (39.5 °C) was observed. *Babesia* spp. in erythrocytes, mild toxic changes in cytoplasm of neutrophils, moderately reactive monocytes and moderate thrombocytopenia were observed on blood smear. The dog showed improvement two days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
49.	At 06/09/2021 mixed breed female dog of 7 years of age was presented to veterinary clinic with a history of moderate injury from encounter with another dog. Mild anemia, moderate neutrophilia and moderate monocytosis were observed on blood smear. Hyperthermia (39.5 °C) was observed. The dog showed improvement three days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
50.	At 05/09/2021 mixed breed female dog of 7 years of age was presented to veterinary clinic with a history of frequent urination from the past few days. Clinical examination of the dog revealed tension in the urinary bladder. Uroliths and pseudopregnancy were diagnosed by ultrasound. The dog was obese. Moderate neutrophilia and mild monocytosis were observed on blood smear. Uroliths were surgically removed from urinary bladder four days after initial appointment. The dog showed improvement two days after surgical treatment. ELISA test of TBEV-specific antibodies was negative.

Table S3. *Continued*

No.	**Data of the breed, age, sex, anamnesis, outcome of the TBE disease and status of TBEV-specific antibodies in blood serum of TBEV RNA PCR-positive dogs**
51.	At 21/10/2020 West Siberian Laika breed male dog of 8 years of age was presented to veterinary clinic with a history of depression, dullness, inappetence, weakness in all limbs and reluctance to walk from the past few days. Hyperthermia (39.9 °C) was observed. *Babesia* spp. in erythrocytes, mild anemia, mild neutrophilia, moderate toxic changes in cytoplasm of neutrophils, moderate monocytosis, moderately reactive monocytes and moderate thrombocytopenia were observed on blood smear. The dog showed improvement on the following day after therapy initiation. TBEV-specific antibodies of 325 NTU/mL were detected by ELISA.
52.	At 02/12/2020 English Cocker Spaniel breed female dog of 8 years of age was presented to veterinary clinic with a history of behavioral changes: standing in fixed position, episodes of altered mental state and anxiety from the past few days. The dog showed improvement two days after therapy initiation. TBEV-specific antibodies of 30 NTU/mL were detected by ELISA.
53.	At 03/06/2020 West Scottish Terrier breed female dog of 8 years of age was presented to veterinary clinic with a history of weakness of hind limbs and difficulties jumping on the bed or climbing stairs from the past two weeks. Clinical examination of the dog revealed tension in the back, lumbar and abdomen muscles. Mild neutrophilia, mild toxic changes in cytoplasm of neutrophils and mild monocytosis were observed on blood smear. Severe periodontitis was observed. The dog showed improvement three days after therapy initiation. Two weeks after the initial appointment, the dog was anxious, excited, hyperactive and yelping. Clinical examination of the dog revealed tension of the back muscles. Mild anemia, moderate neutrophilia, severe toxic changes in cytoplasm of neutrophils, severe monocytosis and highly reactive monocytes were observed on blood smear. One month after the initial appointment the dog was presented to the clinic. Tachypnea, tachycardia and anemic mucous membranes were observed. After X-ray hydrothorax was suspected. Bloody liquid was obtained during thoracentesis. Regardless of intensive care and treatment the dog died. ELISA test of TBEV-specific antibodies was negative.
54.	At 05/11/2021 Papillon breed male dog of 8 years of age was presented to veterinary clinic with a history of reduced activity and reluctance to jump and climb stairs from the past two days. The dog showed improvement on the following day after therapy initiation. TBEV-specific antibodies of 442 NTU/mL were detected by ELISA.
55.	At 05/04/2021 German Shepherd breed male dog of 8 years of age was presented to veterinary clinic with a history of depression, dullness, inappetence and diarrhea from the past three days. Attached tick was found on the body of the dog. Hyperthermia (39.5 °C) and hematuria were observed. The dog showed symptoms of impaired urination two days after initial appointment. Uroliths were not observed in X-ray. The dog showed improvement four days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
56.	At 30/09/2021 German Shepherd breed male dog of 8 years of age was presented to veterinary clinic with severely obtunded mentation. Vomiting was observed for the past week. Intoxication with rodenticide was suspected. The dog died regardless of the intensive care and treatment. TBEV-specific antibodies of 127 NTU/mL were detected by ELISA.
57.	At 23/10/2021 mixed breed female dog of 8 years of age was presented to veterinary clinic with a history of dullness, depression, reduced intake of food, occasional vomiting and excessive panting from the past two days. Hyperthermia (40.5 °C) was observed. *Babesia* spp. in erythrocytes, moderate anemia, leucopenia, reactive lymphocytes, moderately reactive monocytes and moderate thrombocytopenia were observed on blood smear. CaniV4 test was negative. The dog showed improvement four days after therapy initiation. One week after first appointment, pseudopregnancy with presence of lactation was diagnosed. The dog showed improvement five days after treatment initiation. TBEV-specific antibodies of 35 NTU/mL were detected by ELISA.

Table S3. Continued

No.	Data of the breed, age, sex, anamnesis, outcome of the TBE disease and status of TBEV-specific antibodies in blood serum of TBEV RNA PCR-positive dogs
58.	At 12/06/2020 German Spitz breed male dog of 9 years of age was presented to veterinary clinic with a history of inappetence and occasional vomiting from the past two days. Mild anemia, mild neutrophilia and moderately reactive monocytes were observed on blood smear. The dog showed improvement two days after therapy initiation. The dog showed lack of coordination one week after initial appointment. The appetite of the dog was inconsistent. Dysbacteriosis and digestion impairment with malabsorption was diagnosed. The dog showed improvement three days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
59.	At 16/06/2020 mixed breed female dog of 9 years of age was presented to veterinary clinic with a history of vomiting few times a week for the past month. Mild neutrophilia, mild toxic changes in cytoplasm of neutrophils, mild monocytosis and moderately reactive monocytes were observed on blood smear. The dog showed improvement one week after therapy initiation. Cholecystitis was diagnosed one month after initial appointment. ELISA test of TBEV-specific antibodies was negative.
60.	At 15/07/2020 Doberman breed female dog of 9 years of age was presented to veterinary clinic for evaluation of a mass lesion on the front limb. Metastases in the lungs were observed in X-ray. Moderate number of spherocytes, mild reticulocytosis, mild toxic changes in cytoplasm of neutrophils, 14 % of band neutrophils, highly reactive lymphocytes, moderate monocytosis and highly reactive monocytes were observed on blood smear. The dog was euthanized one month after initial appointment. ELISA test of TBEV-specific antibodies was negative.
61.	At 22/04/2020 German Shepherd breed male dog of 9 years of age was presented to veterinary clinic with a history of bleeding of unknown origin from the past four days. Clinical examination of the dog revealed reduced paw replacement of left back limb. Moderate reticulocytosis, moderate number of spherocytes, severe neutrophilia, 21% of band neutrophils, severe toxic changes in cytoplasm of neutrophils, reactive monocytes, lymphocytopenia, reactive lymphocytes and moderate number of aggregates of platelets were observed on blood smear. Hemangiosarcoma was suspected after fine needle aspirate cytology of the spleen. The dog showed improvement four days after therapy initiation. However, the dog was euthanized after onset of respiratory insufficiency and metastases in the heart one month after the initial appointment. ELISA test of TBEV-specific antibodies was negative.
62.	At 01/11/2020 Yorkshire Terrier breed female dog of 9 years of age was presented to veterinary clinic for evaluation of foreign body (bone) which was stuck in the esophagus. The foreign body was removed surgically and the dog showed improvement on the following day after treatment initiation. TBEV-specific antibodies of 30 NTU/mL were detected by ELISA.
63.	At 03/12/2020 Staffordshire Bull Terrier breed female dog of 9 years of age was presented to veterinary clinic with a history of vaginal discharge from the past three days. Mild anemia, moderate leukocytosis, severe toxic changes in cytoplasm of neutrophils, 15% of band neutrophils, severe monocytosis and reactive monocytes were observed on blood smear. The dog showed improvement four days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
64.	At 02/11/2021 mixed breed male dog of 9 years of age was presented to veterinary clinic for evaluation of perineal hernia and rectal diverticula. ELISA test of TBEV-specific antibodies was negative.

Table S3. Continued

No.	**Data of the breed, age, sex, anamnesis, outcome of the TBE disease and status of TBEV-specific antibodies in blood serum of TBEV RNA PCR-positive dogs**
65.	*At 23/10/2020 mixed breed male dog of 10 years of age was presented to veterinary clinic with a history of depression, dullness, inappetence, difficulty standing up, reluctance to walk and hypersensitivity of the back from the past two days. Hyperthermia (40.2 °C) was observed. Moderate anemia, *Anaplasma* spp. morulae in neutrophils, mild neutrophilia, moderate toxic changes in cytoplasm of neutrophils, moderate monocytosis, moderately reactive monocytes and moderate thrombocytosis were observed on blood smear. *Babesia* spp. infection was suspected, although no protozoa were found in erythrocytes and PCR test was not available at the clinic. The dog showed improvement three days after therapy initiation. One week after initial appointment, the dog showed inappetence, dullness, diarrhea, vomiting and hyperthermia (39.4 °C). Blood transfusion was performed. The dog showed improvement two days after blood transfusion. TBEV-specific antibodies of 32 NTU/mL were detected by ELISA.
66.	At 28/10/2020 Golden Retriever breed male dog of 10 years of age was presented to veterinary clinic with a history of constant biting and licking of the right hind limb from the past three days. Hyperthermia (39.4 °C) was observed. The dog showed improvement two days after therapy initiation. TBEV-specific antibodies of 44 NTU/mL were detected by ELISA.
67.	At 02/11/2020 Yorkshire Terrier breed male dog of 10 years of age was presented to veterinary clinic for evaluation of a lump on the right cheek and the swelling of the right side of the face. The dog showed improvement six days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
68.	At 05/12/2020 Miniature Pinscher breed male dog of 10 years of age was presented to veterinary clinic for evaluation of enlarged perianal glands. Clinical examination of the dog revealed enlarged prostate. The dog showed improvement after the therapy was initiated. TBEV-specific antibodies of 454 NTU/mL were detected by ELISA.
69.	At 11/09/2021 Yorkshire Terrier breed female dog of 10 years of age was presented to veterinary clinic with a history of diarrhea, inappetence and occasional vomiting from the past few days. Mild polycythemia, mild neutrophilia and severe toxic changes in cytoplasm of neutrophils were observed on blood smear. The dog showed improvement three days after therapy initiation. TBEV-specific antibodies of 45 NTU/mL were detected by ELISA.
70.	At 06/09/2021 Rottweiler breed female dog of 10 years of age was presented to veterinary clinic with a history of mass lesion on the left hind limb from the past two weeks. Inappetence was observed for about a week. Clinical examination of the dog revealed swollen left tarsus and the dog was reluctant to walk on that limb. Tension and sensitivity of abdomen was observed. Mild leukocytosis and mild monocytosis were observed on blood smear. Cytology of the mass lesion revealed severe inflammation with atypical cells, therefore osteosarcoma/chondrosarcoma was suspected. The dog was euthanized. ELISA test of TBEV-specific antibodies was negative.
71.	At 22/09/2021 Yorkshire Terrier breed male dog of 10 years of age was presented to veterinary clinic with a history of attached tick found on the body of the dog five days prior. The PCR for *Babesia* spp. performed at the clinic was positive. Specific treatment was administered and the dog recovered without showing any symptoms. ELISA test of TBEV-specific antibodies was negative.
72.	At 28/10/2021 Golden Retriever breed male dog of 10 years of age was presented to veterinary clinic with a history of occasional vomiting with blood from the past two days. Mild anemia, mild neutrophilia, 8% of band neutrophils, mild monocytosis and reactive monocytes were observed on blood smear. CRP was 82 mg/L. The dog showed improvement four days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.

Table S3. *Continued*

No.	Data of the breed, age, sex, anamnesis, outcome of the TBE disease and status of TBEV-specific antibodies in blood serum of TBEV RNA PCR-positive dogs
73.	At 16/10/2021 mixed breed female dog of 10 years of age was presented to veterinary clinic with a history of dullness, depression, inappetence and occasional vomiting from the past two days. Pyometra was diagnosed by ultrasound. Mild anemia, moderate neutrophilia, severe toxic changes in cytoplasm of neutrophils, 35% of band neutrophils, reactive monocytes and reactive lymphocytes were observed on blood smear. Ovariohysterectomy was performed six days after initial appointment. The dog showed improvement three days after the surgical treatment was applied. ELISA test of TBEV-specific antibodies was negative.
74.	At 19/10/2021 mixed breed female dog of 10 years of age was presented to veterinary clinic with history of dullness, depression and inappetence from the past few days. Hyperthermia (40.1 °C) was observed. Mild neutrophilia, severe toxic changes in cytoplasm of neutrophils, 12% of band neutrophils, moderate number of reactive monocytes, eosinopenia and moderate thrombocytopenia were observed on blood smear. Three days after initial appointment occasional vomiting was observed. The dog showed improvement four days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
75.	At 22/10/2021 Yorkshire Terrier breed female dog of 10 years of age was presented to veterinary clinic with a history of coughing, inappetence, dullness, depression and lethargy from the past month. The dog was treated specifically for heart failure for more than two years. Clinical examination of the dog revealed hyperthermia (39.7 °C), short breath and heart murmur. Moderate periodontitis was evident. Mild anemia, moderate leukocytosis, severe toxic changes in cytoplasm of neutrophils, moderately reactive lymphocytes and reactive monocytes were observed on blood smear. The dog died at home. TBEV-specific antibodies of 261 NTU/mL were detected by ELISA.
76.	At 16/06/2020 Beagle breed male dog of 10 years of age was presented to veterinary clinic with a history of depression, inappetence, reluctance to walk and difficulties standing up from the past few days. Mild anemia was observed on blood smear. CRP was 159 mg/L. The dog showed improvement four days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
77.	At 27/10/2020 Labrador Retriever breed male dog of 11 years of age was presented to veterinary clinic with a history of depression, dullness and occasional vomiting from the past few days. Two weeks after initial appointment the dog was apathetic and demonstrated excessive panting. Cranial part of the abdomen was sensitive and painful. *Candida* spp. invasion was suspected after gastroscopy. Hyperthermia (39.9 °C) was observed the following day. The dog was hyperactive and excited, standing up and laying down repetitively. In the evening, the dog was anxious, spasmodic pain and shortness of breath were observed. X-ray revealed multiple small mass lesions in the lungs. The dog was euthanized. ELISA test of TBEV-specific antibodies was negative.
78.	At 06/10/2020 mixed breed male dog of 11 years of age was presented to veterinary clinic with a history of depression, dullness and inappetence for three days. Hyperthermia (40 °C) and hematuria were observed. *Babesia* spp. in erythrocytes, moderate toxic changes in cytoplasm of neutrophils, moderately reactive monocytes and severe thrombocytopenia were observed on blood smear. The dog showed improvement two days after therapy initiation. TBEV-specific antibodies of 344 NTU/mL were detected by ELISA.
79.	At 02/06/2020 Yorkshire Terrier breed male dog of 12 years of age was presented to veterinary clinic with a history of occasional vomiting and diarrhea with blood from the past few days. On the second day, the dog showed lack of coordination and reduction of pupillary light reflex. On the third day, menace reflex was reduced and pupils were dilated. Vocalizing without obvious reason was observed on the fourth day. Mild anemia, mild neutrophilia, mild toxic changes in cytoplasm of neutrophils, reactive lymphocytes and moderate thrombocytosis were observed on blood smear. The dog showed improvement five days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.

Table S3. *Continued*

No.	Data of the breed, age, sex, anamnesis, outcome of the TBE disease and status of TBEV-specific antibodies in blood serum of TBEV RNA PCR-positive dogs
80.	At 25/11/2020 French Bulldog breed male dog of 12 years of age was presented to veterinary clinic with a history of seizures (two times within 20 minutes, each lasting about 2 minutes). While at the clinic, the dog experienced a prolonged episode of seizures and was sedated. Seizures did not occur after recovery from sedation, although the vision of the dog was impaired. On the second day, no episodes of seizures or signs of vision impairment were noticed. Repetitive episodes of seizures were observed (three episodes lasting 30–60 seconds each, with periods of 10 minutes in between) on the third day. After seizures, the dog was alert and appetite of the dog was good. On the fourth day, after four episodes of seizures, the dog was disoriented and panting excessively. CSF was clear from cells and bacteria, SG was 1.001, and TBEV RNA was detected by PCR. The dog was anxious, hyperactive, excited and vocalizing on the fifth day. The vision of the dog was impaired, although the appetite was good. The dog showed improvement two weeks after therapy initiation. The episodes of seizures were occasional and specific treatment was continued. Two months later, a long-lasting episode of seizures was observed by the owners. The dog was sedated at the clinic, although the following night after long lasting episode of seizures the dog was disoriented and pupil light reflexes were reduced. The dog was euthanized. ELISA test of TBEV-specific antibodies was negative.
81.	At 19/11/2020 Golden Retriever breed female dog of 12 years of age was presented to veterinary clinic because the owners found the dog nonresponsive. At the clinic, the dog was non-ambulatory and had fever (39.4 °C). Facial reflexes, pupil light reflexes and deep pain sensation were impaired. Mild neutrophilia, moderate toxic changes in cytoplasm of neutrophils and moderate number of basophils were observed on blood smear. On the following day, the state of dog was poor and the dog was euthanized. ELISA test of TBEV-specific antibodies was negative.
82.	At 19/10/2020 Siberian Husky breed female dog of 13 years of age was presented to veterinary clinic with a history of being found by the owners lying on his side. Mild reticulocytosis, moderately reactive lymphocytes, reactive monocytes and mild eosinophilia were observed on blood smear. CSF was free of cells or bacteria, SG was 1.005. The following day prognosis of the dog was poor, the dog was laying on the side, drank water, but had no appetite. The dog was euthanized. TBEV-specific antibodies of 492 NTU/mL were detected by ELISA.
83.	At 11/10/2020 mixed breed male dog of 13 years of age was presented to veterinary clinic with a history of depression, somnolence, inappetence and occasional vomiting from the past three days. Hyperthermia (39.5 °C) was observed. Moderate neutrophilia, mild toxic changes in cytoplasm of neutrophils, 10% of band neutrophils, severe monocytosis and mild thrombocytepenia were observed on blood smear. CRP was 54.4 mg/L. The dog showed improvement six days after therapy initiation. TBEV-specific antibodies of 101 NTU/mL were detected by ELISA.
84.	At 25/09/2021 Yorkshire Terrier breed female dog of 13 years of age was presented to veterinary clinic with a history of depression, dullness, inappetence and occasional vomiting from the past few days. Hypothermia (36 °C) and heart murmurs were observed. Moderate periodontitis was observed. The dog showed improvement three days after therapy initiation. TBEV-specific antibodies of 217 NTU/mL were detected by ELISA.
85.	At 23/10/2021 Chihuahua breed female dog of 13 years of age was presented to veterinary clinic. The dog was presented with a history of increased respiratory rate. Pulmonary edema was diagnosed. Regenerating anemia, mild toxic changes in cytoplasm of neutrophils, mildly reactive lymphocytes, mild thrombocytosis and aggregates of platelets were observed on blood smear. The dog showed improvement two days after therapy initiation. However, one week after initial appointment, the dog was euthanized due to relapse of pulmonary edema. ELISA test of TBEV-specific antibodies was negative.

Table S3. *Continued*

No.	Data of the breed, age, sex, anamnesis, outcome of the TBE disease and status of TBEV-specific antibodies in blood serum of TBEV RNA PCR-positive dogs
86.	At 29/05/2020 Miniature Schnauzer breed female dog of 15 years of age was presented to veterinary clinic with a history of episode of seizures. Uncontrolled urination and disorientation were observed. Moderate reticulocytosis, Jolly bodies in erythrocytes, monocytosis, mildly reactive monocytes and aggregates of platelets were observed on blood smear. On the following day lack of coordination was observed. Anemic mucous membranes and heart murmur were noticed. On the third day, the episodes of seizures were observed. The dog was disoriented, could not walk normally, paw replacement of hind limbs was impaired. Moderately regenerating macrocytic anemia, moderate neutrophilia, severe toxic changes in cytoplasm of neutrophils, mild monocytosis and highly reactive monocytes were observed on blood smear. On the fourth day, after an episode of seizures, the dog had difficulties standing up, coordination was impaired, the dog showed head tilt to the left, the left ear lobe was dropped and the dog was urinating uncontrollably. The dog was euthanized. ELISA test of TBEV-specific antibodies was negative.
87.	At 26/05/2020 Poodle breed female dog of 15 years of age was presented to veterinary clinic with a history of vaginal discharge, lack of coordination and painful back from the past three days. Antimicrobial drugs were administered at another clinic. Mild regenerating anemia, mild neutrophilia, mild toxic changes in cytoplasm of neutrophils and mild monocytosis were observed on blood smear. The dog showed improvement three days after therapy initiation. ELISA test of TBEV-specific antibodies was negative.
88.	At 06/10/2021 mixed breed male dog of 15 years of age was presented to veterinary clinic with a history of neoplastic mass lesion at the base of the tongue. The mass lesion was surgically removed (carcinoma was suspected after cytology). The following day after surgery the dog was weak, hypothermic (36.5 °C) and melena was observed. Regenerating anemia, mild toxic changes in cytoplasm of neutrophils, 6% of band neutrophils and reactive monocytes were observed on blood smear. One week after initial appointment, the dog had insomnia and an episode of seizures. The dog showed improvement seven days after the therapy initiation. The dog was treated with gabapentin for 2 months. ELISA test of TBEV-specific antibodies was negative.

*The virus was isolated in cell culture. Note: morphological blood analysis results were obtained by automated IDEXX Hematology Analyzer and blood smear.

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