LITHUANIAN UNIVERSITY OF HEALTH SCIENCES

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TICK-BORNE ENCEPHALITIS VIRUS: PREVALENCE IN DOMESTIC ANIMAL POPULATIONS IN LITHUANIA AND DEVELOPMENT OF NOVEL SPATIAL SURVEILLANCE STRATEGIES

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LIST OF ABREVIATIONS

°C	–	degree Celsius
µg	–	microgram
µm	–	micrometer
ATCC	–	The American Type Culture Collection
BLAST	–	Basic Local Aligment Search Tool
bp	–	a base pair
CDC	–	Centers for Disease Control and Prevention
cDNA	–	complementary deoxyribonucleic acid
Ct	–	cycle threshold
DNA	–	deoxyribonucleic acid dpi – days post–infection
ECDC	–	European Centre for Disease Prevention and Control
ELISA	–	enzyme–linked immunosorbent assay
EU	–	European Union
EU/EEA	–	European Union and European Economic Area
IgG	–	immunoglobulin G
IgM	–	immunoglobulin M
LIV	–	Louping ill virus
MEM	–	Minimum Essential Medium
MeSH	–	Medical subject headings
MIR	–	minimal infectious rate
mL	–	milliliter
NCBI	–	National Center for Biotechnology Information
nM	–	nanomolar
Nt	–	nucleotide
NUTS	–	Nomenclature of Territorial Units for Statistics
OR	–	odds ratio
p.i.	–	post infection
PCR	–	polymerase chain reaction
POWV	–	Powassan virus
qPCR	–	quantitative polymerase chain reaction
RNA	–	ribonucleic acid
RT–nPCR	–	nested reverse transcription polymerase chain reaction
RT–PCR	–	reverse transcription polymerase chain reaction
SE	–	standard error
TBE	–	tick-borne encephalitis

TBEV – tick-borne encephalitis virus
U – unit
UV – ultraviolet
VNT – virus neutralization test
WHO – World Health Organization
WN – West Nile fever

INTRODUCTION

Tick-borne encephalitis (TBE) is the most important tick-borne viral zoonotic disease in Europe, transmitted through the bites of infected *Ixodes spp*. ticks that serve as main vector and reservoir for the virus [1, 2]. Alimentary infection after consumption of unpasteurized milk of milk products has been recognized as additional route of transmission. Tick-borne encephalitis virus (TBEV) in this route is most commonly transmitted through the drinking of goat milk, however, infection also have been documented through the use of cow or sheep milk [3–9].

The overall number of human clinical cases in Europe has increased in recent years [10, 11]. In Lithuania TBE incidence rates have been increasing by 8.5 % per year for the 45-year period from 1970 to 2014 [12]. Since 2013, the country has the highest incidence rate of the disease in Europe [13]. Moreover, TBE is endemic throughout Lithuania [14].

Targeted disease control measures must be taken on the basis of known virus prevalence. However, this is a major issue. Although the number of autochtonous confirmed clinical TBE cases with a known site of exposure can be used to determine a regional-level TBE risk to some extent, human surveillance alone is insufficient to adequately monitor the prevalence of TBEV as national health authorities in Europe use non-uniform TBE case definitions and risk assessment methodologies [15]. Limitation increases further if wide geographic areas are chosen to estimate the TBEV prevalence because the actual population at risk can deviate substantially from the number of inhabitants in an area due to the focal distribution of the virus [16]. Moreover, vaccine coverage disparities may have an impact on such estimates [17]. Exposure risk can also be influenced by socioeconomic, political, environmental, or climatic factors [10, 18–20]. The flagging and dragging approach for detecting TBEV in ticks is widely used to analyze spatial TBEV circulation [21]. However, this method is also no longer considered reliable, mainly because of low TBEV prevalence rates in ticks and spatiotemporal mismatch between TBEV prevalence in ticks and clinical case notifications in humans [22].

The use of wild or domestic animals as surrogate markers of natural TBEV prevalence recently has piqued interest [23, 24]. In known endemic areas, a significant correlation between seroprevalence in small mammals, dogs, bovids, and cervids and TBE incidence in humans, as well as the capacity of these sentinel species to uncover previously unknown TBEV foci was confirmed [25–29]. However, horses, despite being one of the few species having clinical TBE symptoms [30] have received very little attention as possible

TBEV sentinels, with only a few papers describing the epidemiological significance in TBEV circulation in the nature [30–32].

Testing of milk samples has been proposed as another promising method for determining TBEV prevalence [2]. Milk testing demonstrated significant applicability in mapping high-risk locations in two antibody screening studies in Sweden [33, 34]. To our knowledge, only two investigations aimed specifically at detecting TBEV have been conducted, however, both of which looked at just over a hundred samples [35, 36].

Therefore, overall rationale of the present study was to carry out the investigation on TBEV prevalence in the least studied areas such as horses and small ruminant‘s milk and investigate whether it may help to assess the prevalence of the virus in the environment or may serve as a complement to ongoing monitoring efforts.

Scientific novelty

Here, the prevalence of TBEV in milk of small ruminants was systematically investigated for the first time. The findings revealed a surprising high incidence of TBEV in milk. It has also generated a plethora of new information on the risk factors associated with milk and milk products’ safety. The findings also led to the development of a novel, reliable, non-invasive, and simple-to-use approach for assessing the virus’s presence in nature, which might be used for nationwide TBEV surveillance or be adapted to monitor endemic areas. Results described here includes in-depth analysis of TBEV-specific antibody prevalence in horses. It also includes first report of successful TBEV detection and sequencing results in aforementioned host.

Finally, scientific efforts resulted in two unintended results. First, it was discovered that tick-borne encephalitis virus can be isolated in the MARC-145 cell line, which in turn is a fundamental addition to the existing knowledge about the virus properties, allowing the virus to be isolated in an easy-to-cultivate and low-demanding cell line. Second, the first indirect evidence of probable West Nile fever causative agent circulation in Lithuanian territory was discovered.

The aim of the study

The aim of the study was to determine the prevalence of Tick-borne encephalitis virus in Lithuanian domestic animal populations, assess the safety of animal-derived products and develop animal-based tick-borne encephalitis surveillance system.

Objectives of the study

1. To assess the prevalence of Tick-borne encephalitis in small ruminants, horses and ticks and assess prevalence influencing factors.
2. To evaluate safety of unpasteurized milk and milk products.
3. To develop animal-based tick-borne encephalitis virus spatial surveillance system and validate its effectiveness.

1. LITERATURE REVIEW

1.1. Etiological agent

Tick-borne encephalitis virus is one of the most common arboviruses in Eurasia, circulating between ticks and vertebrates [1]. It is a member of the *Flaviviridae* family, specifically the tick-borne flavivirus group of the genus *Flavivirus* [10]. TBEV genome consist of an 11-kilobyte single-stranded RNA of positive sense that is packed within a 50-nanometer-diameter encapsulated particle [37]. Two viral structural proteins are found in the virus envelope that surrounds the capsid: protein M (membrane), which is produced from a cleaved precursor (PrM), and protein E (envelope). Protein E is the major component of the viral surface and is responsible for producing virus-neutralizing antibodies, which aids in post-infection protection [38]. In addition, TBEV RNA genome encode seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NSlgtv5) [39, 40].

Tick-borne encephalitis virus belongs to the genetically and antigenically related mammalian tick-borne flavivirus group that includes Langat virus (LGTV), Omsk hemorrhagic fever virus (OHFV), Alkhurma hemorrhagic fever virus (AHFV), Kyasanur Forest disease virus (KFDV), Gadgets Gully virus (GGYV), Powassan virus (POWV), Royal Farm virus (RFV), Karshi virus (KSIV), and Louping ill virus (LIV) [40–42]. Only TBEV, LIV and POWV have all been linked to encephalitis in humans and animals, whereas others are mainly associated with hemorrhagic fever [43, 44].

Three classic TBEV subtypes have been identified based on genomic sequence analyses: The TBEV-FE (Far East) virus is found in Asia, primarily in northern China and Russia's east. TBEV-Sib (Siberian) subtype is circulating in the rest of Russia, with an eastern European reach. The major subtype in mainland Europe is TBEV-Eu (European) [45]. Two novel subtypes have been proposed: the Baikalian subtype (TBEV-Bkl), which circulates in the Baikal lake region [46], and the Himalayan subtype (TBEV-Him), which was isolated from Himalayan marmots [47]. Very recently Deviatkin *et al*. [48] suggested a TBEV classification based solely on genetic data, designating seven TBEV subtypes: TBEV-Eu, TBEV-Sib, TBEV-FE, TBEV-2871 (TBEV-Ob), TBEV-Him, TBEV-178-79 (TBEV-Bkl1), and TBEV-886-84 (TBEV-Bkl-2).

Genetic and antigenic variation is low amongst TBEV strains. The maximum degree of genetic variation between main strains within subtypes was 2.2 % at the amino-acid level, and a maximum difference of 5.6 % was detected between the main three subtypes in a comparative study of gene sequences coding for protein E, which is similar to the range of variation reported for other flaviviruses [45, 49].

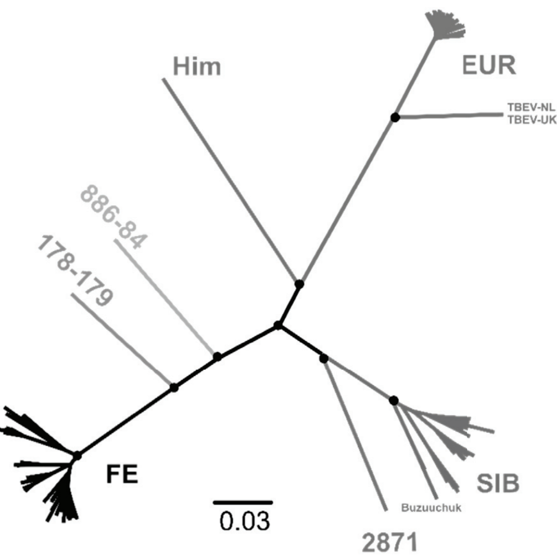

__Fig. 1.1.1.__ Unrooted maximum likelihood tree for Tick-borne encephalitis virus (adapted from Deviatkin et al. [48]).

1.2. Ecology

1.2.1. The tick vectors

Hard ticks are the primary arthropod vectors for TBEV transmission. *Ixodes ricinus* is the most common tick vector in Europe which is directly associated with European TBEV subtype, while *Ixodes persulcatus* is the most common tick vector in Russia and Asia, associated with Siberian and Far Eastern subtypes [50–54]. 22 more tick species have been found to be capable of carrying the virus to date [55–59]. However, their contribution to virus circulation is very limited with exception of *Dermacentor reticulatus* [60–62]. Although it rarely bites human beings, *Dermacentor reticulatus* ticks outnumber *Ixodes* tick bites on large domestic and game animals, perhaps resulting in an additional TBEV circulation cycle [63, 64].

Ticks can become infected while feeding on viraemic hosts or while co-feeding with an infected tick [18,19]. Interestingly, co-feeding guarantees virus transmission even when the host is already have immunity against TBEV [65]. Effective virus transmission can occur through the simultaneous feeding of an infected tick and uninfected ticks in close proximity on the same animal [66]. Ticks in this framework are thought to be their own reservoir hosts, employing the animal to which they are attached as a transmission bridge [67].

Furthermore, the virus can be transmitted vertically from an infected adult female tick to her progeny by transovarial transmission but this appears to be ineffectual and unimportant for the virus's maintenance cycle [68, 69]. Non-viraemic transmission between co-feeding ticks, which mainly occurs between tick nymphs and larvae, is thought to play a critical part in the TBEV transmission cycle [66, 70]. It is particularly important, when animal host's viraemia is mild or of short duration, or when the host develops TBEV-specific immunity [65, 71]. Therefore, the close ecological relationships of *I. ricinus* and *I. persulcatus* ticks with their vertebrate hosts, which result in larvae and nymphs co-feeding on the same hosts, may explain why these two tick species are considered the principal TBEV vector ticks [72].

The virus is also maintained in the tick population through trans-stadial transmission, which means that the virus is carried by the infected tick throughout its entire life cycle, which includes eggs, larvae, nymphs, and adults [67]. Given to its vast population, the nymph appears to be the most crucial stage for virus transmission [56]. Tick saliva contains chemicals that are analgesic, anti-inflammatory, and anti-coagulant, allowing the tick to feed undetected [73]. TBEV, therefore, potentially might circulate from an infected egg to an adult tick and then back to its eggs through this inner population circulation [74]. However, recent evidence suggesting transmission rates between stages are not as great as expected [75]. In addition, to aforementioned TBEV transmission routes, there are some evidence regarding transmission through sexual intercourse [1].

Climate and environmental changes have contributed to a significant extension of the occurrence range of ticks with high epidemiological importance [63, 76–80] as well as an increase in their population size [81] throughout Eurasia during the last few decades. Moreover, ticks appear to have adapted to lower temperatures and may be partially active in winter months [82, 83]. Ticks may infect the host with flaviviruses soon after attachment to the skin and the initiation of blood intake [84].

1.2.2. The mammalian hosts

Susceptibility to TBEV in host vertebrates varies depending on species. Some have a high viraemia and so play a significant role in TBEV transmission to ticks. A good reservoir host would be an animal infected with TBEV that keeps the virus circulating in its bloodstream for as long as feasible, in titers high enough to infect a feeding tick without dying from disease, allowing more ticks to feed on it and become infected [67].

Rodent species can act as reservoir hosts by sustaining viraemia for a long time. TBEV virus was detected for up to 168 days after experimental infecti-

on in bank voles [85, 86]. Moreover, for a minimum of three months, infectious TBEV can be isolated from brain, kidney, and spleen of experimentally infected common voles [24]. In addition, they perhaps are able to transmit the virus vertically to progeny [87, 88]. Therefore, rodents such as red vole, bank vole, common vole, European pine vole, yellow-necked mice, wood mouse and striped field mouse are believed to serve as both amplifiers and bridge hosts [67]. They all appeared to be in regular contact with TBEV, displaying antibodies and positive RT-PCR results in varying degrees across Europe [24, 25, 89–93].

TBEV antigen of TBEV-specific antibodies were detected in variety of large animal species, including cattle [94, 95], sheep [27, 95], goats [27, 96, 97], few cervid species [98–100], wild boars [100], dogs [101–103] and horses [30, 31]. However, following TBEV infection large animal species as well as human beings typically show low or nonexistent viraemia and only indirectly contribute to the virus's persistence by hosting ticks [104–106].

In addition, few anecdotal TBEV transmission routes have been reported, including blood transfusion [107], organ transplantation [108], transmission from a viraemic mother to her progeny through breast milk [109], aerosol transmission among laboratory personnel [110, 111] and after the slaughtering of a viraemic goat [112].

1.2.3. Birds

The role of birds in TBEV circulation and their capacity to act as reservoir for the virus is yet unclear. However, birds are thought to play a key role in the spread of the virus to new endemic areas, as is the case with many other tick-borne infections [113]. TBEV appears to be able to infect a variety of bird species, primarily forest passerines [114]. Birds' capacity to readily cross barriers like rivers and motorways allows them to transmit attached ticks to new locations where animals dwelling on the forest floor might not be able to reach [115]. However, the virus it carries could be spread if the translocated tick finds a suitable location with the correct climatic and fauna parameters [116]. Nevertheless, dispersion over larger distances appears unlikely for TBEV, as *Ixodes* ticks have a relatively short feeding period, lasting up to nine days. This results in early detachment and short distance travelled while attached to the bird [117]. Summary of TBEV transmission cycle is presented in Fig. 1.2.1.

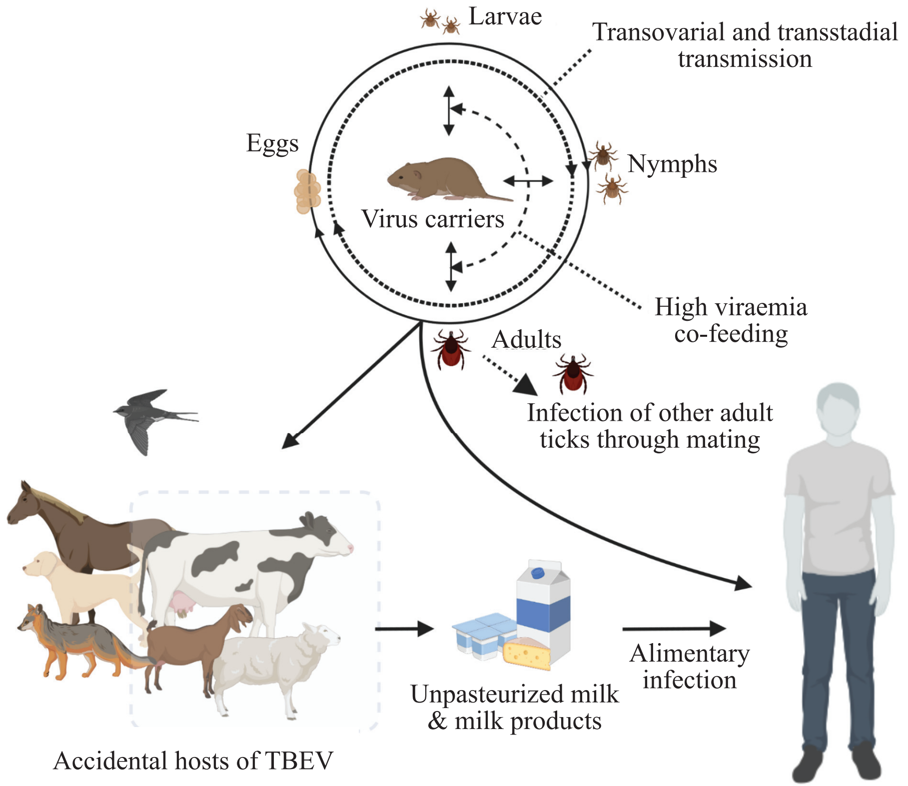

Fig. 1.2.1. *Tick-borne encephalitis virus transmission and maintenance in the tick vector and its hosts.*

Ticks become infected with TBEV mostly while feeding on an infected rodent or insectivore at early developmental stages, either through co-feeding with an infected tick or through the presence of high quantities of virus in the animal's blood. The virus survives all of the tick's embryonic phases and is transmitted transovarially from adult to egg. Large mammals and birds act as host for adult ticks and sustain infected and susceptible tick populations, however due to low levels of viraemia, they are poor TBEV transmitters. Tick bites are the most common route of human infection, but unpasteurized milk from infected ruminants can also transmit TBEV to people. Visualization was created with accordance of the following sources: [2, 60–62, 72, 85, 86, 118].

1.3. Clinical manifestations

Published data suggest that 70–98 % of TBE infections are asymptomatic [119, 120]. Nevertheless, exact proportion is hard to access as patients showing mild clinical symptoms may remain undiagnosed [121]. Disease severity and mortality has strong association with TBEV subtype. The Eu-

ropean subtype causes moderate encephalitis with a fatality rate up to 2 %. The Far-Eastern subtype causes severe disease that can result in 20–30 % mortality. Siberian subtype causes a milder form of encephalitis than Far-Eastern subtybe, with fatality rates ranging from 3 to 6 % depending on the particular area [40, 122–124].

Typically, incubation period ranges from 7 to 14 days [104]. When caused by the European TBEV subtype, the disease has a typical biphasic course in about 75 % of patients. The first phase is characterized by non-specific symptoms such as moderate fever, headache, myalgia, lethargy, general malaise, anorexia, nausea, and others [120]. This phase lasts 2 to 7 days and is followed by improvement or an asymptomatic period of about 1 week. The second phase then emerges, which is characterized by meningitis in nearly half of adult patients, meningoencephalitis in about 40 %, and meningoencephalomyelitis in around 10 % of patients [125, 126]. The majority of patients with a monophasic course of the disease have central nervous system involvement resulting in meningitis and meningoencephalitis while a small percentage of patients have a febrile illness with headache but no meningitis [121].

Chronic neuropsychiatric or neurological sequelae, such as depression, lack of focus, or paresis of the face or limbs owing to chronic myelitis or encephalitis, are seen in 10–20 % of individuals with severe disease [118]. The involvement of cranial nerves in TBE patients has been also reported. Hovewer, it has favorable prognosis in the majority of cases [127–129]. Hyperkinesis of the limbs and face muscles, lingual tremor, convulsions, and respiratory muscle paresis are some of the other symptoms. Death might occur as soon as one week following the onset of clinical symptoms [2].

TBE when caused by alimentary transmission has few differences in the course of the disease. Contrary to classical transmission, incubation period after exposure via the alimentary route, is shorter and usually taking 3–4 days [104], but it might be as little as two days [6]. During the second phase of milk-borne TBE, non-severe meningoencephalitis is more common. Clinical signs of tick-associated TBE, such as meningitis or meningoencephalitis are frequently more severe [130]. Patients who recover from milk-borne infections have a nearly 100 % chance of recovering without neurological complications, but tick-borne TBE is more frequently associated with long-term disability and mortality [40]. Finally, TBE following a tick bite is usually sporadic, whereas alimentary infections are more commonly related with family incidence or minor outbreaks linked to the intake of raw milk products [6, 72].

Following TBEV infection, animals may show a variety of clinical symptoms. While most wild animals are asymptomatic, encephalitis can occur in certain species as bank voles. *Macaca sylvanus* monkeys on the other hand,

are showing similar symptoms to those seen in humans [10, 26]. Clinical manifestations in domestic animals, such as cattle, sheep, goats, and pigs, are very rare [131–133] or not reported [100, 105, 134]. TBE has been reported in horses with clinical indicators such as poor general health, anorexia, ataxia, abrupt cramping, convulsions, and paralysis of the neck and shoulder muscles, though reports are scarce [30–32]. Infection of the CNS can also cause fever and neurological symptoms in dogs. However, in dogs TBEV infection is likely underdiagnosed [135, 136].

1.4. Epidemiology

1.4.1. Brief overview

TBEV is circulating in central, northern, and eastern Europe, Russia, and the Far East, which includes Mongolia, China and Japan [104]. TBE clinical cases are estimated to number between 10 000 and 12 000 per year worldwide [137], with more than 3000 human TBE cases hospitalized each year in Europe [120]. Furthermore, a considerable percentage of TBEV infections are considered undiagnosed [126].

The distribution of TBE clinical cases is very patchy. The chance of contracting an infection after being bitten by a tick ranges from 1/200 to 1/1000, depending on geographic location of where the bite took place [56].

The number of human clinical cases has increased in recent years; nonetheless, there have been significant fluctuations in the notification rates [10, 11]. This could be explained by the complex combination of social and environmental factors, including the virus's simultaneous spread into new geographical areas as a result of changes that favor tick or host reservoirs. It also could due to the increased exposure to infected ticks as a result of socioeconomic and political changes affecting outdoor leisure habits, or increased harvesting of wild vegetables and fruits due to economic constraints [10, 19, 20]. For example, a recent research in Scandinavian countries have found that women perceive risk differently than males, are more likely to employ protective measures, and have a better understanding of tick-borne diseases [138–140]. Increased medical awareness and more sophisticated diagnostics also may play a role [10, 18].

TBE cases are most common in the warm months of April to November, which is also when tick activity is at its peak [104]. In Central Europe, where *I. ricinus* is the most common tick species, there is a two-peak distribution of TBE cases (first in June and July, second in September and October), whereas in the Ural region, Siberia, and the Far East, where

I. persulcatus is common, cases are most common in May and June [40]. Men are afflicted more commonly than women in all age groups [129, 141, 142].

It is important to mention that TBE has become a more global problem as tourism has grown. According to TBE surveillance data available in Austria, the overall risk of contracting TBE for a non-vaccinated tourist staying in a highly endemic region for 4 weeks during the TBEV transmission season is estimated to be around 1 case per 10 000 person-months of exposure, which is about the same as the risk of contracting typhoid fever or malaria while traveling in India [143, 144].

1.4.2. Current situation

In the EU, TBE became notifiable disease in 2012, and the current case definition was established in 2018 [145]. From 2015 to 2019, the EU TBE notification rate ranged between 0.4 and 0.7 cases per 100 000 people. Lithuania, Czech Republic and Estonia had the highest notification rates in 2019, as they had in prior years. [146] From 2015 to 2019, the notification rate in Czechia and Norway has steadily increased [146]. The majority of cases are still diagnosed during the summer months, with no evidence of a significant seasonal shift [147].

A possible case of TBE was discovered in southern England in July 2019. This is the first report of a possible TBE case in a human in the United Kingdom [148]. TBEV was discovered in ticks in southern England in September 2019, after previously only being seen in ticks in northern England [149, 150].

Based on latest data available [146], 3246 TBE cases were confirmed in EU/EEA countries in 2019. That accounted for 0.7 cases per 100 000 population. Case fatality was 0.7 %. There were no cases recorded in only three countries: Greece, Luxembourg and Romania.

Lithuania had the highest notification rate (25.4 cases per 100 000 people), followed by Czech Republic (7.3) and Estonia (7.3). Czech Republic (n = 773), Lithuania (n = 711), and Germany (n = 445) reported the highest number of confirmed cases in 2019. Summary of confirmed TBE cases both in numerical and epidemiological frames is presented in Figure 1.4.1, which was generated based on the official data extracted from ECDC surveillance Atlas [151].

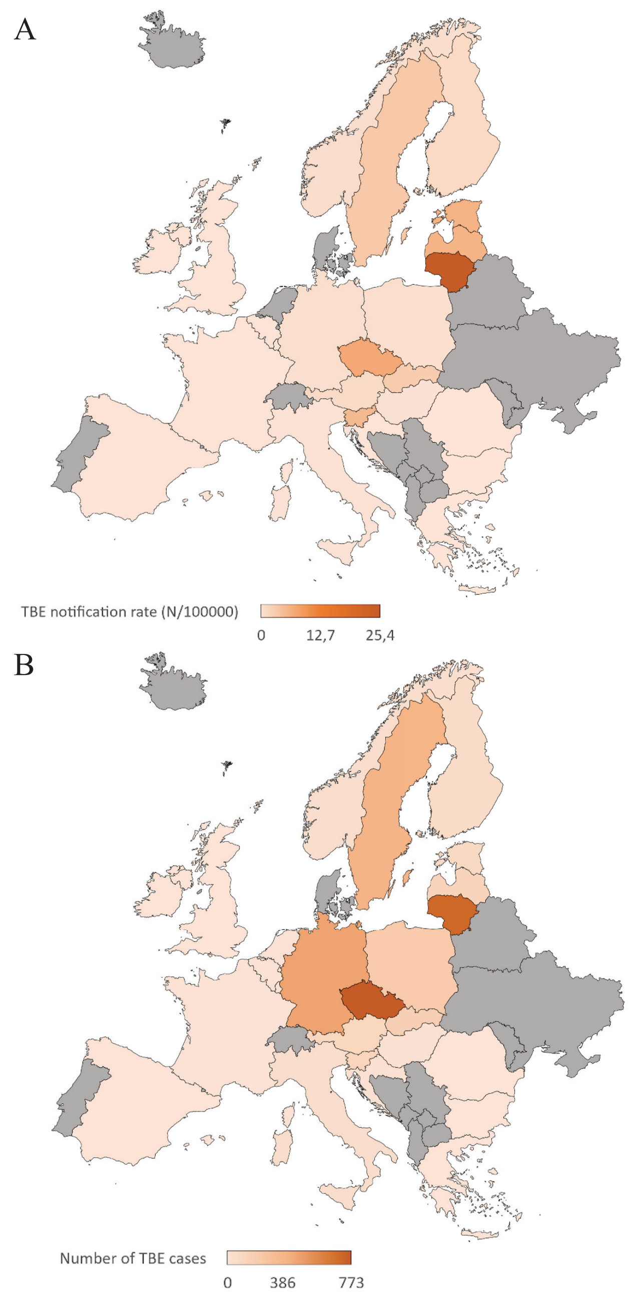

Fig 1.4.1. *Distribution of confirmed tick-borne encephalitis cases in humans in Europe in 2019.*

Note: TBE rates per 100 000 population (A), number of confirmed clinical TBE cases (B). Gray color indicates countries that have not provided data or are not members of the EEA. Graph was produced using data extracted from ECDC Surveillance Atlas [151].

Interestingly, notification rate in 2019 increased compared to the consistent rate of 0.6 recorded in the preceding three years. The highest difference was observed in Lithuania. The notification rate here has increased significantly since 2018, from 13.7 to 25.4 cases per 100 000.

1.8 % (n = 54) of all confirmed cases were travel-related. Eleven countries have reported imported cases, with Germany having the greatest number (n = 22). 39 patients were reported to have been infected in EU Member States, and 2 cases had a travel history in Russia and India. The place of infection of remaining cases was not specified due incompleteness of the data provided.

The seasonality of reported cases in 2019 was confirmed, with 95 % of verified cases reported between May and November. 720 cases were reported at the 2019 peak in July, representing for 22 % of confirmed cases. The bimodal distribution that was observed in 2017 and 2018 was not present in 2019 (Fig. 1.4.2.).

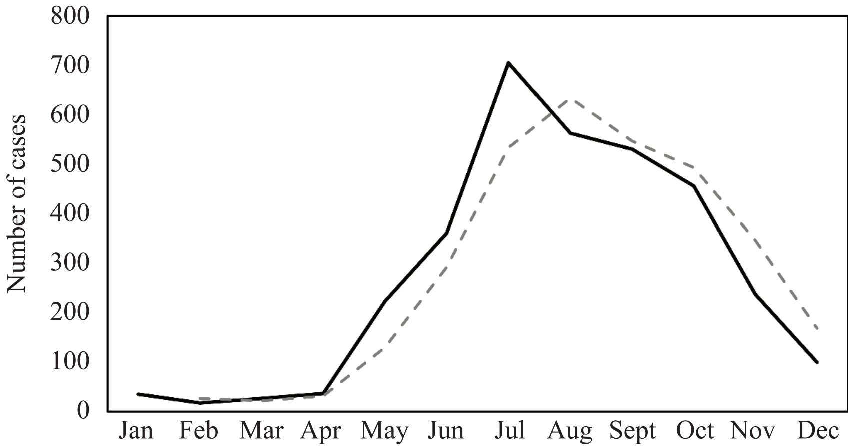

Fig. 1.4.2. *Distribution of confirmed tick-borne encephalitis cases in humans in a monthly scale, EU/EEA, 2019*

Note: Black line – actual data of 2019; dash line – moving average model. Graph was produced using data extracted from ECDC Surveillance Atlas [151].

1.4.3. Alimentary infections

It has been suggested that the alimentary route is responsible for about 1 % of all TBE infections in humans [118, 121, 152]. However, it appears that infections through this route are more common. Some food-borne TBEV infections are asymptomatic or mildly symptomatic, and thus may be undiagnosed

[153]. In as many as 50 % of all documented cases of human TBE in Germany, the patients had no recollection of the tick bite episode prior to the onset of the disease, implying that some of the cases were caused by food-borne TBEV transmission [154]. Despite the fact that TBE is a notifiable disease in the European Union, there is no officially confirmed strategy that allows to methodically register and assess foodborne TBE outbreaks in a country or EU-level. As a result, global knowledge of alimentary TBE infection is mainly based on individual reports from researchers or particular institutions.

A summary of all available data [3–7, 72, 91, 152, 154–167] on food borne TBE outbreaks is presented in Figure 1.4.3. Graph was created using data extracted from Medline/PubMed, Google Scholar and Scopus databases using respective keywords and MeSH terms. Data extraction procedure was performed with accordance of Cohrane guidelines for Systematic Reviews [168]. As it is shown in the map, alimentary TBEV infections have been recorded in at least ten European countries, mostly in Eastern and Central Europe, however the proportion of infection varies by country. The greatest recorded numbers of TBE alimentary cases are in Hungary and Slovakia. It is important to sound a note that former foodborne outbreaks are often discussed in the Lithuanian professional environment. However, no publications on this have been published so far. Therefore, the information provided on the map should be evaluated with caution, as the real prevalence pattern may differ and include more countries.

Unpasteurized milk and milk products are thought to have a higher nutritional value than pasteurized milk by certain customers. As evidenced during the investigation of a food-borne TBE outbreak [152], their main motivation for drinking unpasteurized milk was to provide a balanced diet for their family. The largest number of proponents of unpasteurized milk appears to be made up of producers and their family members [169]. However, metropolitan regions have seen an increase in the use of such products as well. In recent years, the unpasteurized milk or its products have been sold not only in marketplaces, but also in vending machines where locals and tourists can purchase raw dairy products [170]. The number of cases is likely to increase in the future as the spread of alimentary TBE virus infection may be aided by an increase in worldwide milk consumption [171, 172]. This is especially true in Europe as 24 of the top 30 countries with the largest per capita consumption of milk and dairy products are in this continent [173].

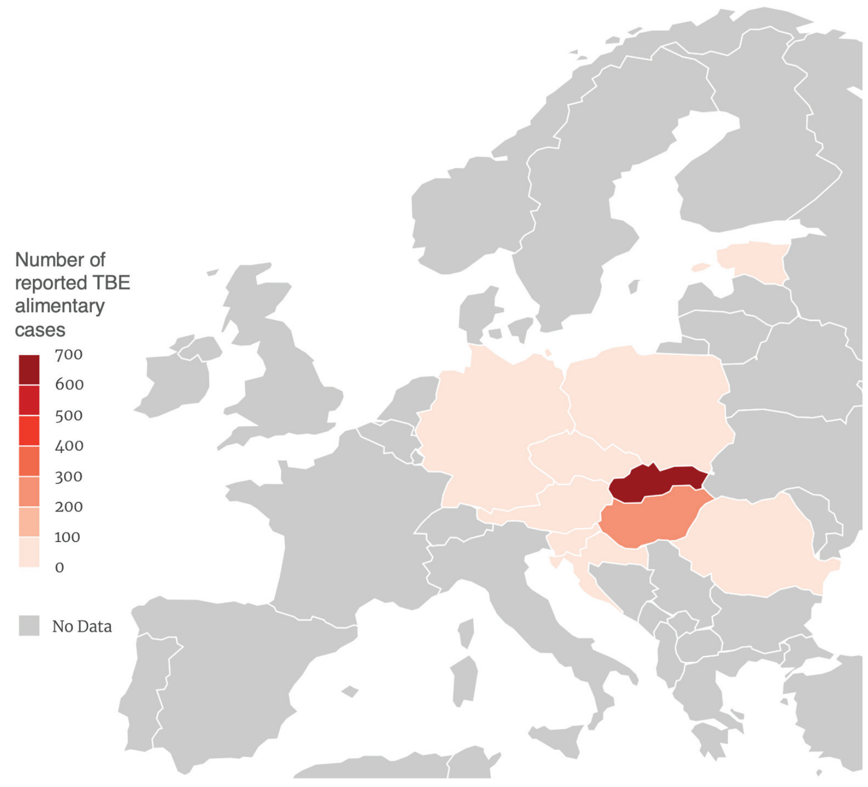

Fig.1.4.3. *Number of reported TBE alimentary cases in Europe.*

Note: Graph was produced using data extracted from following sources [3–7, 72, 91, 152, 154–167].

1.5. Animal-based TBEV surveillance systems

Many animal-based alternatives for TBEV prevalence assessment were proposed in recent years. Most of them showed many shortcomings, however, few have proven the potentiality at some degree to be adopted as surveillance system.

Although a high number of positive samples are found in wild rodents, these results have no statistical association with TBE incidence in humans [24, 25, 92, 174, 175]. The distribution of positive samples also does not coincide with the boundaries of the known endemic zone. Legal concerns with animal trapping permits and natural protection acts, as well as labor-intensive collecting and analysis of huge sample sizes are additional limitations [23].

TBEV-specific IgG antibodies survive for a long time in dogs [135, 136]. Seroconversion rates are frequently reported to be high [29, 102, 103]. Howe-

ver, no correlations with human TBE incidence have been found with exception of one study [99]. Furthermore, the animals' undefined territorial radius can impair the accuracy of the spatiotemporal prevalence estimates.

Cervid species demonstrate a brief viraemia and a low-titre antibody response after infection [176]. Moreover, extensive haemolysis and blood sample contamination are common serological study problems [23, 176]. In the context of matching human TBE risk, prevalence results in cervid species are frequently conflicting and mutually exclusive [98, 124, 177–181].

A single study on wild boars found an association between TBE incidence in humans and wild boars [180]. However, among other wildlife-related and previously noted drawbacks, their foraging range is limited, necessitating the collection of a significant number of samples.

Horses have long persistence of TBEV-specific antibodies [23, 30], and their pastures are generally stable, which can aid in extrapolating results for a prevalence assessment model. In the case of ruminants, the situation is very similar. In addition to favourable location and extended persistence of IgG antibodies, ruminants are characterized by high viremia, which is even much greater and longer detectable in milk than in blood [182]. Moreover, as mentioned earlier, studies in ruminants lead to accurate TBEV spatial prevalence risk assessment [33, 34].

2. MATERIALS AND METHODS

2.1. Sample collection and sampling sites

2.1.1. Horse serum sample collection

301 horse serum samples were collected in 32 stables according to cross-sectional study design in May 2019 at the peak of tick questing period. Sampling stratification was applied to overcome the tendency of random sample to either over-represent or under-represent sections of the sampling frame. NUTS3 level administrative units were considered as strata. The number of samples from each stratum was determined by proportional allocation and was estimated to represent Lithuanian horse population with a 5 % margin of error (95 % CI). The total sampling frame was defined using national database provided by National Land Service under the Ministry of Agriculture of the Republic of Lithuania.

Stables were included to the study if met additional criteria: horses had a well-defined territorial radius, were turned out to the pastures every day and were not vaccinated against any flaviviruses. To perform TBEV risk factor analysis stable-level information (herd size, ration composition, pasture time) and animal-level information (gender, age, breed, prior travel and health records) were collected.

Serum samples of 3–4 mL were obtained from selected horses with regard to animal welfare regulations. Then the serum samples were centrifuged and stored at −20 °C until further use.

2.1.2. Milk sample collection

A total of 1363 goat and 312 sheep milk samples were collected according to a longitudinal study design. Due known short TBEV viremia period, milk samples were collected periodically once every 4–5 days from bulk milk tank throughout tick activity and lactation periods lasting between April and November. Samples were collected for two seasons in 2018 and 2019.

Sampling frame was based on National Livestock Register database of Lithuania. The same sampling stratification technique was applied as for the collection of equine serum samples discussed above. The criteria that had to be met in order to be included in the study were the following: goats and sheep were not vaccinated against any flaviviruses and not acaricide-treated; farms were located not in the urban area and did not apply consistent tick control measures in their territorial radius. Samples were collected from farms of

various sizes (small – 1–5; middle-size – 6–20 and large – ≥21 milk producing animals) according to the data in the aforementioned database.

Milk samples of 10–15 mL in volume were obtained from milk bulk tanks and stored frozen at –20 °C until further use.

2.1.3. Sample collection of unpasteurized milk products

To assess survival of tick-borne encephalitis in goat milk products, 247 cheese (sour milk cheese, n = 82; cheese aged 30 days (± 5 days), n = 81; cheese aged >60 days, n = 84) and 42 yogurt samples were collected.

A 300 mg sample was formed from each cheese for molecular analysis. Each such sample consisted of a pooled sample from several different locations on the same cheese. The inclusion in the sample of the outer part of the cheese in contact with the environment where exposure to viral genetic material is least likely due to exposure to UV rays has been avoided. Samples of 300 μL of yogurt were formed from different locations without prior mixing.

All samples were obtained directly from farmers or purchased at markets. The samples represent the production of 18 different manufacturers. As these samples were only intended to assess the safety of the products and were not intended to assess the geographical distribution of the tick-borne encephalitis virus, the geographical coordinates reflecting the place of production are not detailed.

2.1.4. Tick collection

A total of 2925 ticks were collected. 2684 questing ticks were collected by flagging and dragging method in the close vicinity of the farms where milk samples were obtained. Remaining 241 ticks were collected directly from equine host. All ticks were collected once per season as it typically done in studies of this nature. Questing ticks were collected in both 2018 and 2019 at a single point in time between April and November. Ticks from equine hosts were collected during the same visit when horse serum samples were collected.

Tick pools were formed depending on the development stage, sex, species and sampling site. Up to 10 *Ixodes ricinus* adults; 20 nymphs; 50 larvae or 5 *Dermacentor reticulatus* were grouped per pool. After collection, ticks were maintained alive until reaching laboratory. Then ticks were dissected and homogenized in phosphate-buffered saline, inserted into liquid nitrogen and then ground into a fine powder in a mortar. Each homogenized suspension was centrifuged, supernatant was collected and stored at −20 °C until further use.

2.2. Data on human TBE incidence

Raw data on confirmed human TBE cases was obtained from Centre for Communicable Diseases and AIDS. Human population data in NUTS3 level was obtained from the Lithuanian Department of Statistics

2.3. ELISA and Virus neutralization test

Equine serum samples were tested by ELISA for presence of TBEV IgG using the EIA TBEV IgG kit (TestLine Clinical Diagnostics, Czech Republic) according to the manufacturer's instructions. The results were calculated as the negative control/sample ratio. A < 150 % was used as a cutoff value for negative samples and 200 %> for positive samples.

To avoid possible cross-reactivities with other flaviviruses all horse serum samples were retested by Virus neutralization test (VNT). The TBEV-specific neutralizing antibodies were assessed using gold standard in-house neutralization assay. Complement was inactivated before testing by heating 30 min. in water bath at 56 °C. Samples were then diluted from 1/5 to 1/320 in Minimum Essential Medium (MEM). The serum dilutions were incubated with 100 $TCID_{50}$ of TBEV strain obtained after 6 passages of cultivation on Vero cell culture (ATCC® CCL-81™). Cells were then assessed for the presence of cytopathic effects at 3, 5 and 7 days post-infection (p.i.). TBEV reciprocal titre of ≥1/20 was considered as positive in VNT.

In addition, samples were retested by ELISA test for West Nile virus IgG using WN Competition Multi-species Ig kit (ID.vet, France).

2.4. TBE virus detection

Viral RNA from 300 μL of skimmed milk, centrifuged tick or cell supernatant or serum was exctracted using the GeneJET RNA Purification Kit (Thermo Scientific, USA) according to the manufacturer's instructions. Samples were tested by RT-PCR and confirmatory conventional PCR for the presence of TBEV-specific RNA using primer sets provided in detail in table 2.4.1.

Table 2.4.1. *Primers used in the study*

Primers/probe	Primer sequence (5' to 3')	Reference
F-TBE 1	GGGCGGTTCTTCTCC	[183]
R-TBE 1	ACACATCACCACCTCCTTGTCAGACT	
TBE-WT	FAM-TGAGCCACCATCACCCAGACACA-TAMRA	
TBE NCR5F	GCGTTTGCTTCGGA	[184]
TBE NCR5R	CTCTTTCGACACTCGTCGAGG	
TBE NCR5Fn	CGGATAGCATTAGCAGCG	
TBE NCR5Rn	CCTTTCAGGATGGCCTT	
TBE NS5F	GAGGCTGAACAACTGCACG	[185]
TBE NS5R	GAACACGTCCATTCCTGATCT	
TBE NS5Fn	ACGGAACGTGACAAGGCTAG	
TBE NS5Rn	GCTTGTTACCATCTTTGGAG	
F-16s-Ixodes	AAAAAAATACTCTAGGGATAACAGCGTAA	[183]
R-16s-Ixodes	ACCAAAAAAGAATCCTAATCCAACA	
16s-Ixodes-Probe	TTTTGGATAGTTCATATAGATAAAATAGTTTGC GACCTCG	

TBEV-specific RNA was amplified in 25 μL a total reaction mixture containing of 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 conditions comprised of reverse transcription at 42 °C for 30 min, denaturation at 94 °C for 10 min, followed by 45 cycles at 95 °C for 15 sec and 60 °C for 1 min.

Samples positive by real-time PCR were prepared by one-step RT-PCR and nested PCR for sequencing of NCR or NS5 region. RT-PCR amplification was carried out in a reaction volume of 25 μl that contained the following mix of reagents: 12.5 μL of 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 target RNA. The cycling conditions comprised cDNA synthesis 30 min at 42 °C, denaturation for 5 min at 96 °C, followed by 40 cycles for 30 sec at 96 °C, 30 sec at 40 °C and 30 sec at 68 °C for NS5 fragment and 40 cycles for 1 min at 92 °C, 1 min at 37 °C and 1 min at 72 °C for NCR5 fragment.

The nested PCR amplification was performed 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 primers (260 nM), 18 μL of nuclease-free water and 5 μL of target RNA. The cycling conditions were an initial denaturation for 5 min at 96 °C, followed by 40 cycles for 30 sec at 96 °C,

30 sec at 40 °C and 30 sec at 68 °C for NS5 fragment and 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C for NCR5 fragment. All reactions were carried out in triplicate set frame.

Quality of RNA extraction of tick samples was assessed using RT-qPCR targeting 16s rRNA of I.ricinus. Reaction was performed in 25 µL a total reaction mixture containing of 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 (200 nM) of each forward and reverse primer and 1.5 µL (100 nM) probe. The cycling conditions comprised of reverse transcription at 42 °C for 30 min, denaturation at 94 °C for 10 min, followed by 45 cycles at 95 °C for 15 sec and 60 °C for 1 min.

Each PCR product was loaded into a 1.5 % TopVision agarose (Thermo Scientific) gel containing 1xTAE buffer and ethidium bromide (5mg/mL) for 55 min. The GeneRuler 100 bp and 50 bp DNA ladders were used as the molecular size marker. Products were then purified with GeneJET PCR Purification Kit (Thermo Scientific, USA) according to the manufacturer's instructions.

2.5. Sequencing and Phylogenetic Analysis

Sequences, including reference TBEV strains Neudörfl (U27495); Sofjin (AB062064) and Vasilchenko (AF069066) were retrieved from the GenBank database and aligned manually using ClustalW software (Clustal, Dublin, Ireland) in MEGA X package. The neighbor-joining method was used for phylogenetic tree construction with 1000 bootstrapping replicates.

2.6. Virus Load Quantification

Viral quantification assay has been modified based on a previously published study. A fragment corresponding to the amplified NS5 region of the TBEV was cloned into the pJET1.2 vector using the CloneJET PCR Cloning Kit (Thermo Scientific, Waltham, MA, USA) and Transform-Aid Bacterial Transformation Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Plasmid DNA extraction and purification was then performed using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, Waltham, MA, USA) and quantified using the Qubit dsDNA BR Assay Kit (Invitrogen, USA) according to supplier's protocol. Standard curves were generated after 10-fold dilutions of stock DNA. Serial dilutions served as templates for qPCR reactions. The starting concentration of the sample was expressed in arbitrary fluorescence units and was converted to the number of

genome copies/mL. A total of 40 repeated measurements were made on each diluted sample to calibrate the final standard curve, presented in Fig. 2.6.1.

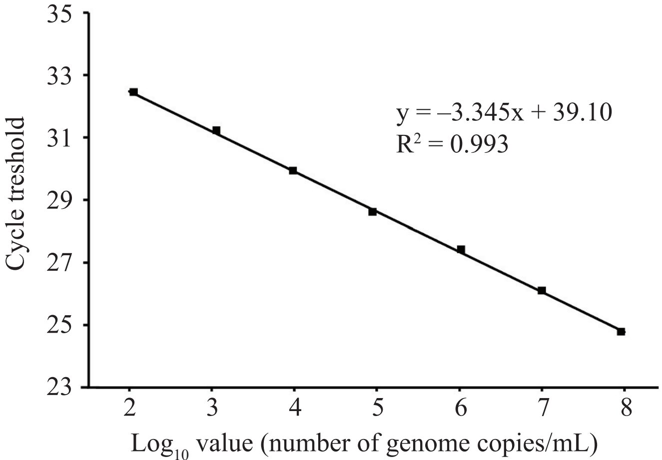

Fig. 2.6.1. *Standard curve of qPCR using serial dilutions of stock DNA.*

Quantification was based on Real-time PCR using SYBR Green I dye (Thermo Scientific), nested NS5 primer set and respective cycling conditions described above. All the samples were tested in triplicates and mean values of viral copy equivalents were calculated.

2.7. Virus Isolation

To assess the viability to infect cells and obtain sufficient number of viral copies necessary for sequencing positive samples were isolated in Vero (ATCC® CCL-81™) and MARC-145 (ATCC® CCL-12231™) cell lines. Cells were maintained in Minimum Essential Medium (MEM, Gibco, USA) containing 10 % heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 100 U mL^{-1} penicillin and 100 μgL^{-1} streptomycin at 37 °C in 5 % CO_2 and air mixture.

Selected samples were passed through a 0.22-µm pore size microfilter (Techno Plastic Products AG, Trasadingen, Switzerland) for purification. Cells were then inoculated in 25 cm^2 tissue culture flasks (TPP Techno Plastic Products AG, Trasadingen, Switzerland) for 1h at 37 °C, after which infection mixture was replaced by 10 mL of Minimum Essential Medium.

The negative control consisted only of MEM and 100 U mL^{-1} penicillin and 100 µg L^{-1} streptomycin.

Cells were examined for the occurrence of cytopathic effect through 6 serial passages which were performed in triplicates including triplicate of posi-

tive and negative controls for each round of analysis. The success of isolation was assessed by RNA extraction and RT-PCR as described above.

2.8. Statistical Analysis

Exact binomial method was used to calculate confidence intervals. Binary logistic regression analysis, odds ratio (OR), regression coefficient (β), standard error (SE) and chi-square test were utilized to test the significance of the risk factors. Pearson correlation was used to evaluate association of human and animal TBEV prevalence. The minimal infection rate (MIR) was calculated as the ratio of the number of positive pools to the total number of ticks tested. MIR = number of positive pools/total number of ticks tested × 100 %. Differences on a dichotomous dependent variable between two related groups were assessed by MnNemar's test. All statistical analysis and mapping were performed using the programming language R. Maps were build using following libraries: plyr, ggplot2, lattice, rgdal, rgeos, sp, maptools, maps and grDevices.

2.9. Funding

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3. RESULTS AND DISSCUSION

3.1. TBEV prevalence in milk of small ruminants

The presence of TBEV RNA was tested in 1363 goat and 312 sheep unpasteurized bulk milk samples collected from 17 goat and 4 sheep farms. Overall, TBEV was found in 62 (4.5 %) and 14 (4.5 %) goat and sheep bulk milk samples, respectively. However, due longitudinal sample collection design, there were two cases of three consecutive positive goat milk samples per farm. As this was likely to occur, time intervals between positive samples and their viral load were measured. Therefore, based on the progressive decline in viral load these instances were identified as two separate infections. Consecutive positive samples attributable to a single infection was thus omitted from further statistical analysis. As a result, the total number of TBEV positive samples was adjusted to 58/1363 (4.3 %, 95 % CI 3.3–5.5) for goat and 14/312 (4.5 %, 95 % CI 2.5–7.4) for sheep samples. All remaining positive samples were indisputably assigned to single infection whereas an interval of at least 10 days was observed between the nearest positive samples.

Specificity of all PCR-positive samples were confirmed by partial genome sequencing based on NCR fragment of TBEV. Sequences have been submitted to GenBank under accession numbers: MZ664211-MZ664256. BLASTN search (NCBI Genbank) revealed that all sequences obtained from milk samples were 89.2–94.1 % similar to the reference strain of the European subtype of TBEV (Neudörfl; Genbank U27495). In contrast, sequences obtained from milk samples showed low degree of similarity to Siberian or Far Eastern subtypes of TBEV. Thus, unlike in neighboring Estonia and Latvia, where all three main subtypes have been co-circulating for almost two decades [76, 186], only the European subtype is circulating in Lithuania.

It is important to note, that sequences obtained in this study did not cluster with other Lithuanian sequences. This could be explained by the fact that total number of Lithuanian sequences uploaded to databases is rather small. In addition, most of them are based on E or NS3 encoding segments. Therefore, further whole genome sequencing efforts are needed to gain better understanding of regional TBEV genetic diversity. This is especially important because a recent study revealed that TBEV genetic diversity within certain geographic areas is far more complex than previously thought, resulting in relatively high nucleotide-level differences in samples collected in a small geographical area [187].

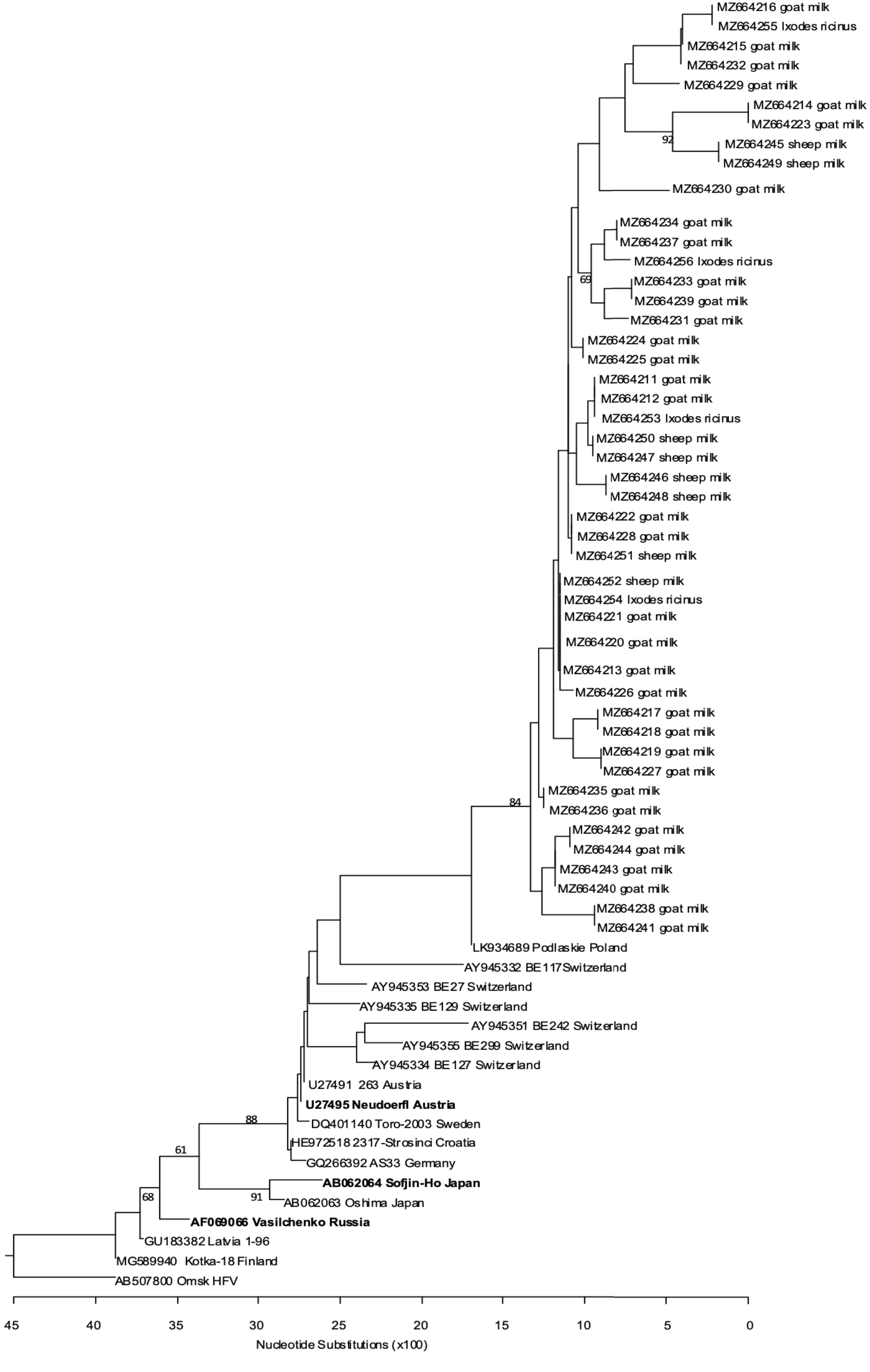


Fig. 3.1.1. *Phylogenetic tree of TBEV sequences obtained from milk samples and ticks collected in the vicinity of the farms.*

In 2018 and 2019 at least one positive sample was found in 70.6 % and 64.7 % of tested goat farms, respectively. During both years of the investigation, at least one positive sample was found on 75 % of sheep farms. TBEV prevalence patterns in sheep milk samples were similar as in goat milk samples. There were no statistically significant differences between species in any of the risk factor tested.

These results are consistent with the epidemiological studies on the alimentary TBEV outbreaks which occur mainly from the consumption of goat's milk and a little less frequently, sheep‘s milk [3, 5–7, 188, 189]. Based on the prevalence and population size of dairy cows and the amount of milk consumed, the number of outbreaks after consuming cow's milk is low [190]. This is confirmed by a study conducted in Lithuania, but not yet published, in which 495 bulk milk samples collected in various locations of the country were examined and none of them were found to be positive for tick-borne encephalitis virus.

Although the prevalence of TBEV was shown to vary with time and over the two lactation periods (Fig 3.1.2.), no significant temporal period was found. A temporal effect was also not determined in the study conducted in Lithuania in 2017–2019 which was focusing on the prevalence of TBEV in *Ixodes Ricinus* and *Dermacentor reticulatus* ticks [191].

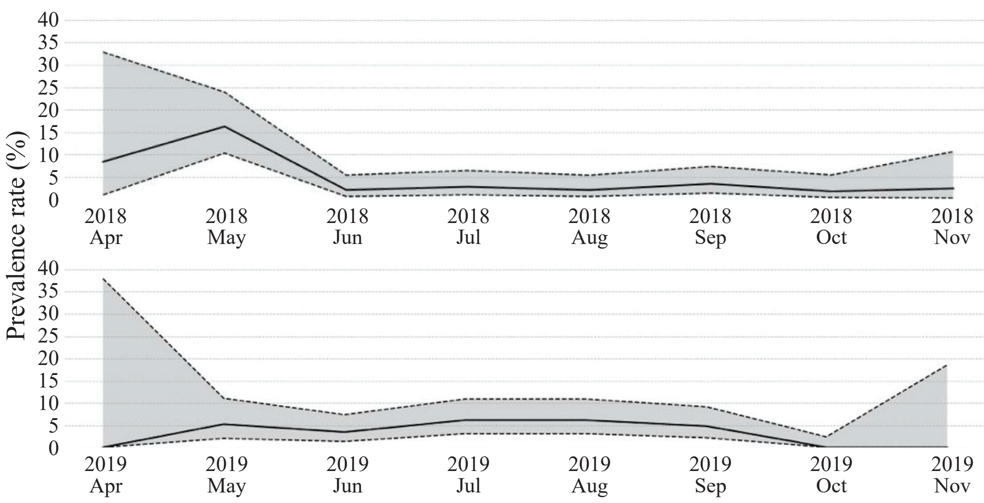


Fig. 3.1.2. *Longitudinal distribution of TBEV positive cases in goat and sheep milk samples in 2018 and 2019.*

Nevertheless, a temporal analysis revealed a substantial link between virus prevalence in animals and TBE incidence rates in humans in 2019 (Fig. 3.1.3.). Unfortunately, in 2018, the same dependence (r = 0.65) was not

statistically significant, although it did demonstrate a trend toward significance ($p = 0.083$). The association was not observed in 2018 potentially due to the remarkably high TBEV prevalence rate in May, which in turn could potentially be explained by the exceptional climatic and environmental conditions favourable to the tick development. The human behaviour also may play a role. It is reasonable to infer that individuals do not visit nature as frequently in May, and hence are less likely to be exposed to ticks. The fact that most of the clinical cases of the disease occur in the summer and early fall [192, 193] supports this explication. More research is necessary to find out whether this one-month case is a single phenomenon or recurring periodically. However, two-year data imply that TBEV prevalence assessment based on milk samples is preferable to current approaches based on tick screening as these results do not correlate with increased human risk [191, 194, 195]. That is particularly important to emphasize, as relationship between these dependences is one of the most crucial elements in assessing whether or not this form of prevalence assessment is reliable [22].

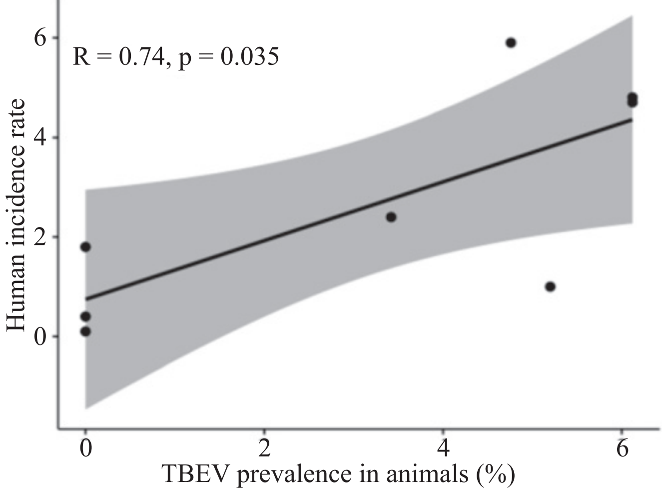


Fig. 3.1.3. *Linear correlation in longitudinal scale between TBEV prevalence in animals and incidence rate in humans in 2019.*

Figure 3.1.4. shows a summary of milk sample collection sites and TBEV spatial distribution. The geographic coordinates of each farm were randomly shifted up to 0.1° from their true positions to guarantee anonymization and personal data protection. Spatial analysis showed that positive samples were uniformly dispersed throughout Lithuania's territory. Therefore, there was no statistical relationship showing that any particular locality is at higher risk of TBEV.

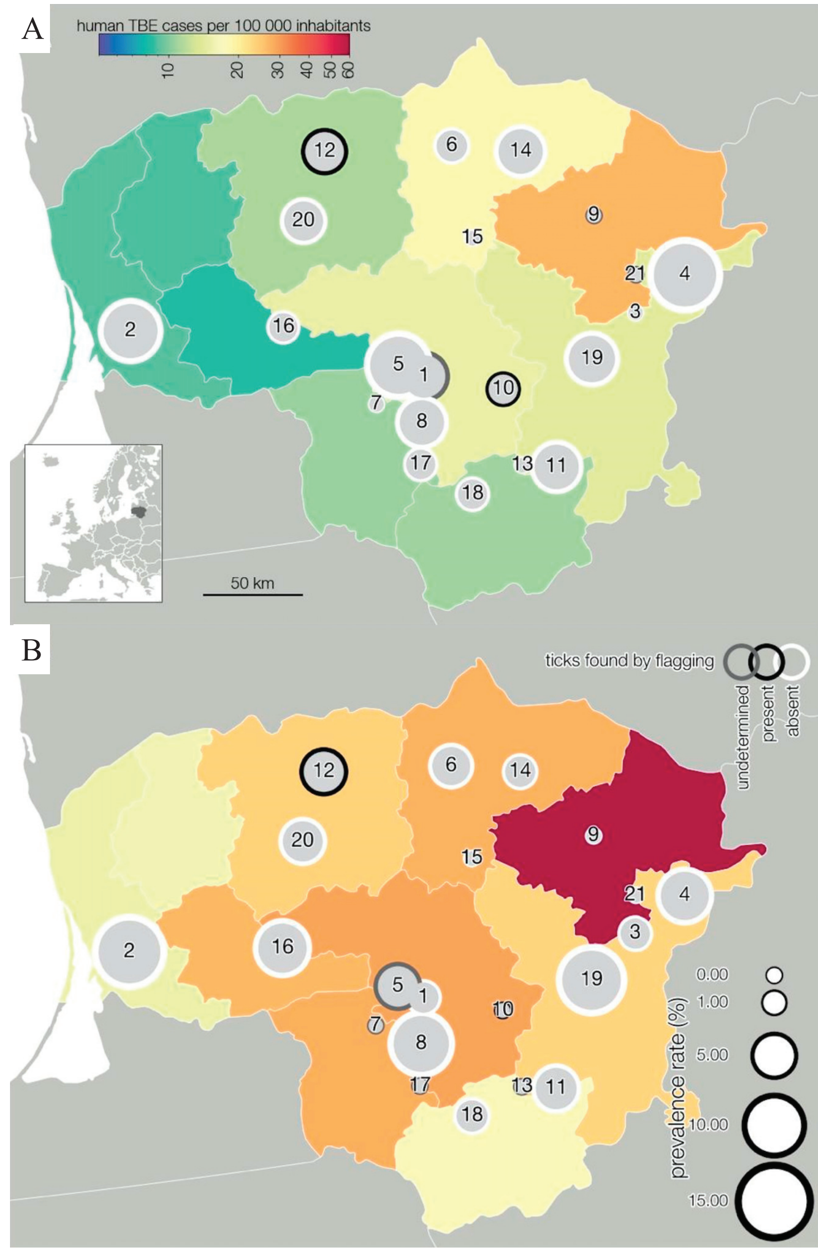


Fig. 3.1.4. *Geographical location of the milk sample collection sites and distribution of TBEV positive cases.*

Note: 2018 results (A), 2019 results (B). The size of the circles represents the TBEV prevalence rate in a particular farm. Goat farms are numbered 1–17, whereas sheep farms are numbered 18–21. Colored administrative units indicate human TBE cases at NUTS3 level.

Interestingly, farm size (6–20 > 1–5 > more than 21 animals) was found to have a non-linear relationship with TBEV prevalence ($r = 0.54$, $p = 0.01$). Moreover, a linear association between viral load and the volume of milk produced on the farm was also observed ($r = 0.64$, $p < 0.005$). The findings of viral loads are shown in Figure 3.1.5. More detailed information, including data on milk yield and number of animals per farm is shown in Table 3.1.1. However, farm ID referring to the map in Figure 3.1.4. is masked by number mixing due to personal data protection.

These are hitherto unknown results with practical significance. As the results show, the size of the farm is of great importance in terms of the potentiality to shed the virus to the milk. It can be added that these results were plausible, although never directly confirmed - most outbreaks, as their analyzes show, occur after the consumption of milk or milk products produced on a small farm [3, 5]. To date, to the best of our knowledge, no outbreak from a large farm has been confirmed.

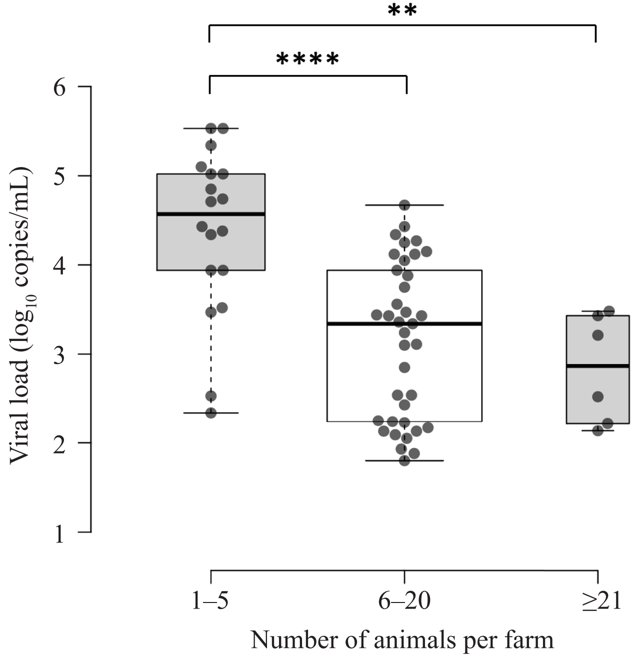


Fig. 3.1.5. *Viral load expressed as log_{10} viral RNA copies/mL.*

Asterisks and horizontal lines at the top of the graph indicate statistically significant differences (** $p < 0.01$; **** $p < 0.0001$).

Table 3.1.1. *Milk amount per bulk tank in tested farms*

Farm ID	Milk amount per bulk tank (liters)	Number of animals per farm	Number of TBEV positive samples	TBEV viral load (log_{10} copies/mL)		
				Mean	Min	Max
1	2.4	4	2	4.1	3.5	4.7
2	1.8	3	8	4.3	2.3	5.5
3	6	10	4	3.0	2.0	4.1
4	28.2	47	0			
5	21	15	4	2.3	1.8	3.1
6	11.2	8	10	3.7	3.2	4.3
7	56	40	1	2.2		
8	12.6	9	8	2.8	1.8	4.1
9	11.2	8	6	3.1	2.0	4.2
10	19.6	14	3	2.0	1.8	2.2
11	5.6	4	0			
12	4.2	3	7	4.4	3.4	5.5
13	77	55	0			
14	84	60	1	2.5		
15	8.4	6	5	2.6	1.9	4.1
16	28	20	4	3.0	2.4	3.3
17	224	160	0			
18	30.8	22	4	3.0	2.1	3.5
19	77	55	0			
20	8.4	6	4	4.2	3.4	4.6
21	11.2	8	1	3.1		

Note: 1–4 – sheep farms; 5–21 – goat farms. Data on farm ID referring to the map in Fig. 3.1.4 are not provided due to personal data protection.

To confirm the presence of infective virus, all positive samples were inoculated on Vero and MARC-145 cells, which were then evaluated for cytopathic effect (CPE), following confirmation by PCR. Overall, 13/58 (22.4 %, 95 % CI 12.5–35.2) goat milk suspensions were successfully isolated and caused CPE beginning 4–6 days post-infection (p.i.) (Fig. 3.1.6). Only one TBEV isolate was successfully isolated from sheep milk.

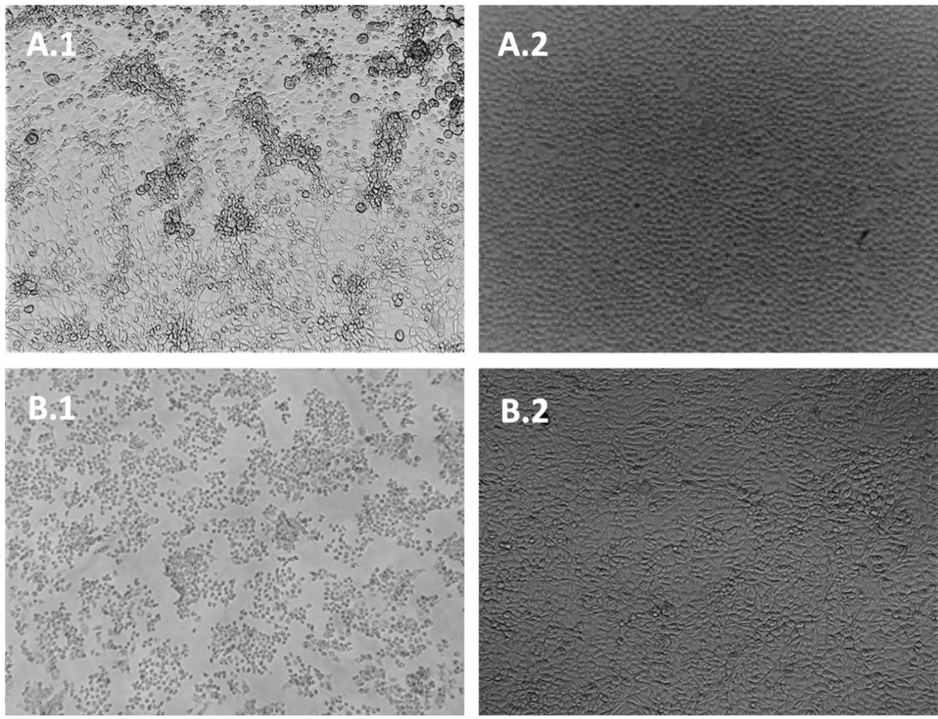


Fig. 3.1.6. *Photomicrographs of virus isolation studies showing characteristic of cell lysis as marker of CPE (unstained, magnification: 50X).*

Note: MARC-145 (A), VERO (B), CPE in cell line (1), Control group (2).

Because no equivalent studies have been conducted, these findings cannot be compared to those of other epidemiological research. These results, on the other hand, are in line with experimental data that show the virus may survive for two days in milk at room temperature before rapidly decreasing in titer and eventually becoming undetectable [196]. Although our findings indicate that the virus does not persist for a long time and that the majority of postive samples are incapable of infecting cells and thus potentially cause disease, incidences of infection after consuming dairy products demonstrate that the virus under certains circumstances can persist for a much longer time. Unfortunately, the factors determining long-term survival have not been systematically studied and are largely unknown

The virus load changed significantly depending on the number of passages, demonstrating 1–3 $\log_{10}$ increase at the sixth passage (Fig. 3.1.7.). Although the expression of the cytopathic effect and the virus load varied, the results did not differ significantly between the two cell lines. However, to the

best of our knowledge, this is the first study to demonstrate that TBEV can be isolated in a MARC-145 cell line, which in turn is a fundamental addition to the existing knowledge about the virus characteristics, allowing the virus to be isolated in this easy-to-cultivate and non-demanding cell line.

In summary, only two studies aiming to detect the TBE causative agent in milk have been performed to date [35, 36], both of which were limited in terms of sample size. The findings presented here are the most complete, revealing an unexpectedly high TBEV prevalence and a wide geographic distribution, as well as a wealth of information on risk variables not elucidated in any study before.

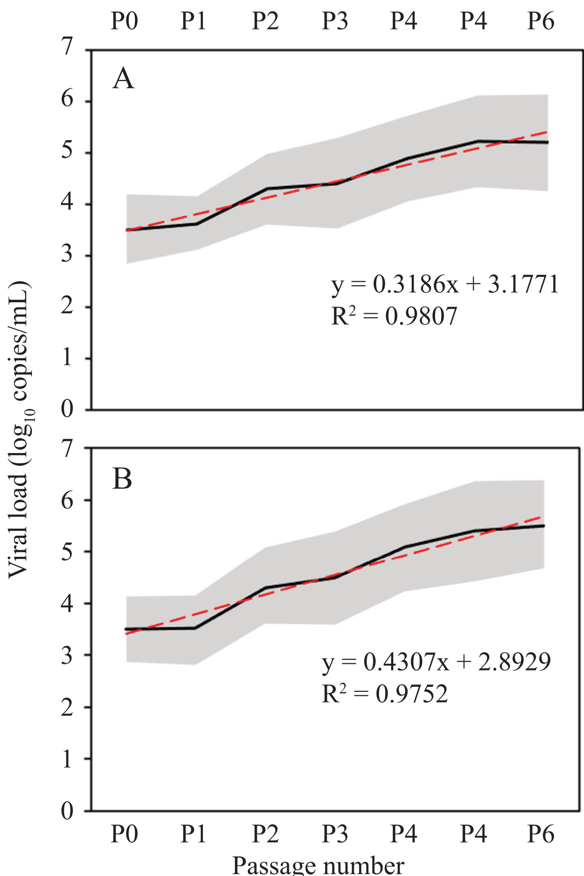


Fig 3.1.7. *Mean changes in viral loads in Vero (A) and MARC-145 (B) cell lines over 6 serial passages.*

Note: Best-fit line is shown as red dash line.

3.2. TBEV detection in milk products

Of the 247 cheese samples varying in degree of maturation and 42 yoghurt samples, only 3 were tested positive for tick-borne encephalitis virus. All three positive samples were detected in sour milk cheese. Positive samples accounted for 1 % (95 % CI 0.2–3.) of all dairy products tested. Partial genome sequencing was used to confirm the specificity for TBEV. Phylogenetic analysis showed that all detected TBEV strains belong to the European subtype (Fig. 3.2.1.) None of the positive samples were isolated in cell culture. Comparative summary of samples on milk or it's products is shown in Figure 3.2.2.

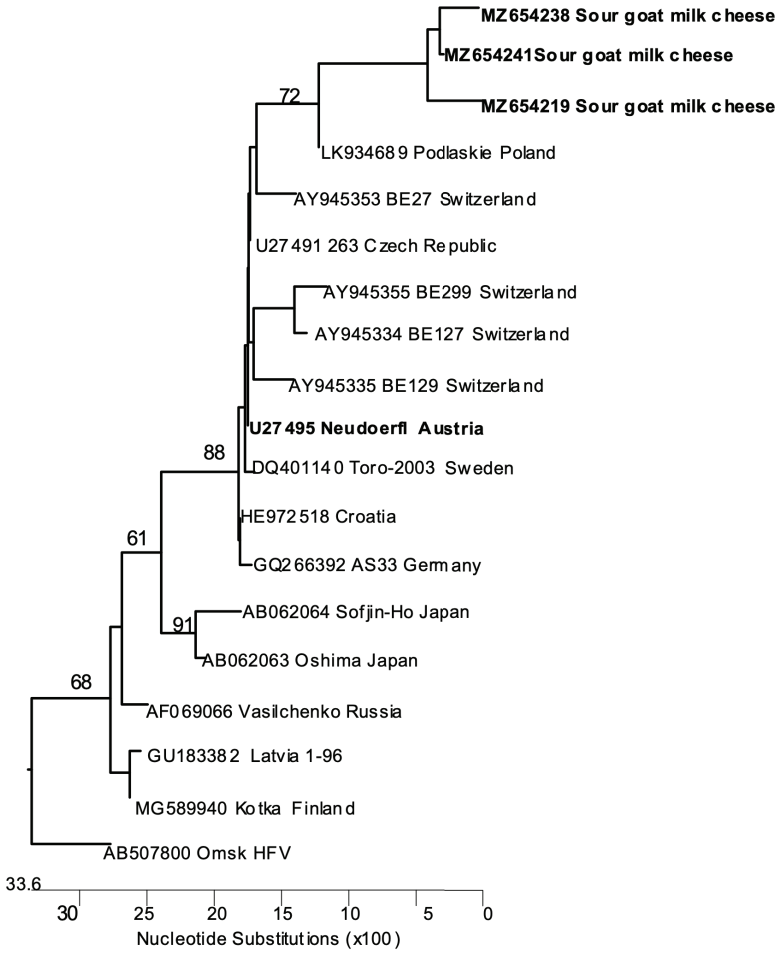


Fig. 3.2.1. *Phylogenetic tree of TBEV sequences obtained from milk products.*

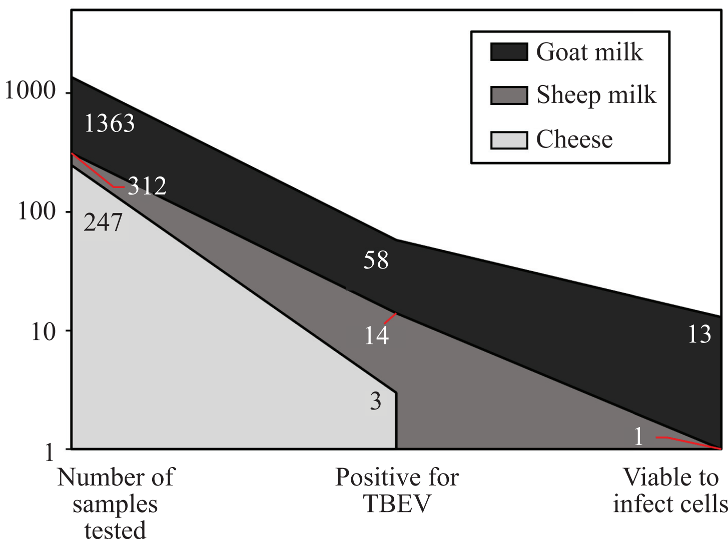


Fig. 3.2.2. *Comparative summary of the samples on the milk and milk products.*

Due to the relatively small sample size, generalizing conclusions cannot be drawn. However, there is a clear tendency for the virus to be detected only in the least technologically processed and least matured cheeses, and even in these the virus cannot be isolated indicating that the viral structural composition is already lost. Given that the RNA enveloped virus's structure renders it to be sensitive to temperature changes [72], this is a likely result.

Currently available scientific evidence based on experimental studies indicates that virus is not destructed if the temperature during the preparation does not reach 65 °C for at least 30 minutes [197]. Another study showed that although the simulation of cheese fermentation have reduced virus levels, the virus was still detectable as a residual [196]. The virus load measurement in this study is line with aforementioned findings. The mean viral load per milliliter in cheese samples was 2.1 $\log_{10}$ copies. As shown in Figure 3.2.3. it was significantly lower than the results obtained from milk samples.

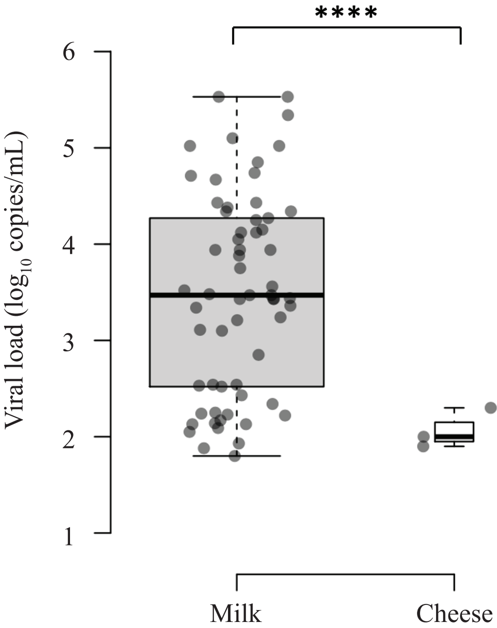


Fig. 3.2.3. *Viral load expressed as log_{10} viral RNA copies/mL.*

Asterisks and horizontal lines at the top of the graph indicate statistically significant differences (**** $p < 0.0001$).

Although there is a lack of experimental results to pinpoint the situation, it can be speculated that in longer-maturing cheeses the virus eventually loses its structure and becomes non-infectious due to longer exposure to temperature and UV light. Thus, products with a longer technological process are likely to be significantly safer against the tick-borne encephalitis virus.

3.3. TBEV prevalence in horse serum

Direct viral detection in large herbivores is limited due to a brief period of viremia during which flaviviruses are eliminated from serum, and to our knowledge, we are the first to report successful PCR and sequencing results in horses. The only study we know of that reported a single sample potentially positive by PCR was conducted in Germany [30]. However, their attempts at sequencing and culturing were unsuccessful.

TBE virus in the present study was found in 12 (3.9 %; 95 % CI 2.3–6.8) of all examined equine serum samples (n = 301). All sequences of equine serum were 93.0–97.1 % identical to the reference strain of the European subtype (Neudörfl; Genbank U27495), according to a BLASTN search (NCBI Genbank). The similarity with the Far Eastern subtype (Sofjin; AB062064)

and the Siberian subtype (Vasilchenko; AF069066) reference strains, on the other hand, was 78.3 % and 84.4 %, respectively (Fig. 3.3.2.). The sequences have been submitted to the GenBank database under the accession numbers MT981174-MT981178 and MW187721-MW187725.

Despite earlier published results showing that milk has a considerably higher viral load than blood [198, 199], our findings demonstrated the contrary (Fig. 3.3.1.). The explanation of the results is related to the study's design. In this investigation, serum samples were taken from individual horses, whereas milk samples were collected from a bulk milk tank. As a result, an undetermined dilution effect appears to be present in pooled milk samples. Therefore, despite their differences, our findings do not invalidate the findings of earlier investigations.

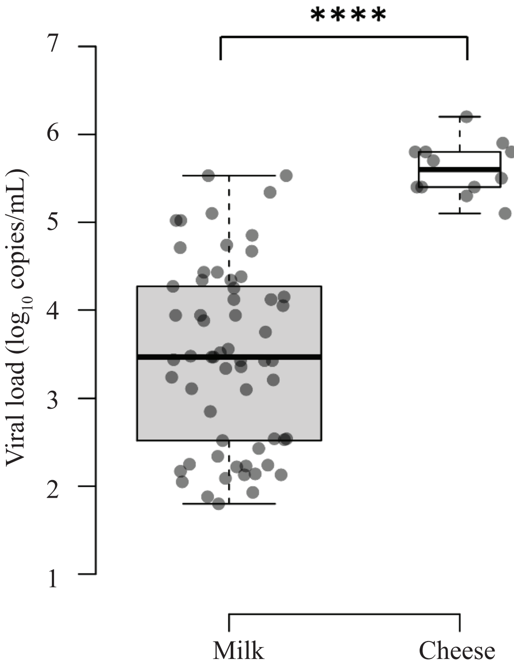


Fig. 3.3.1. *Viral load expressed as $\log_{10}$ viral RNA copies/mL.*

Asterisks and horizontal lines at the top of the graph indicate statistically significant differences (**** $p < 0.0001$).

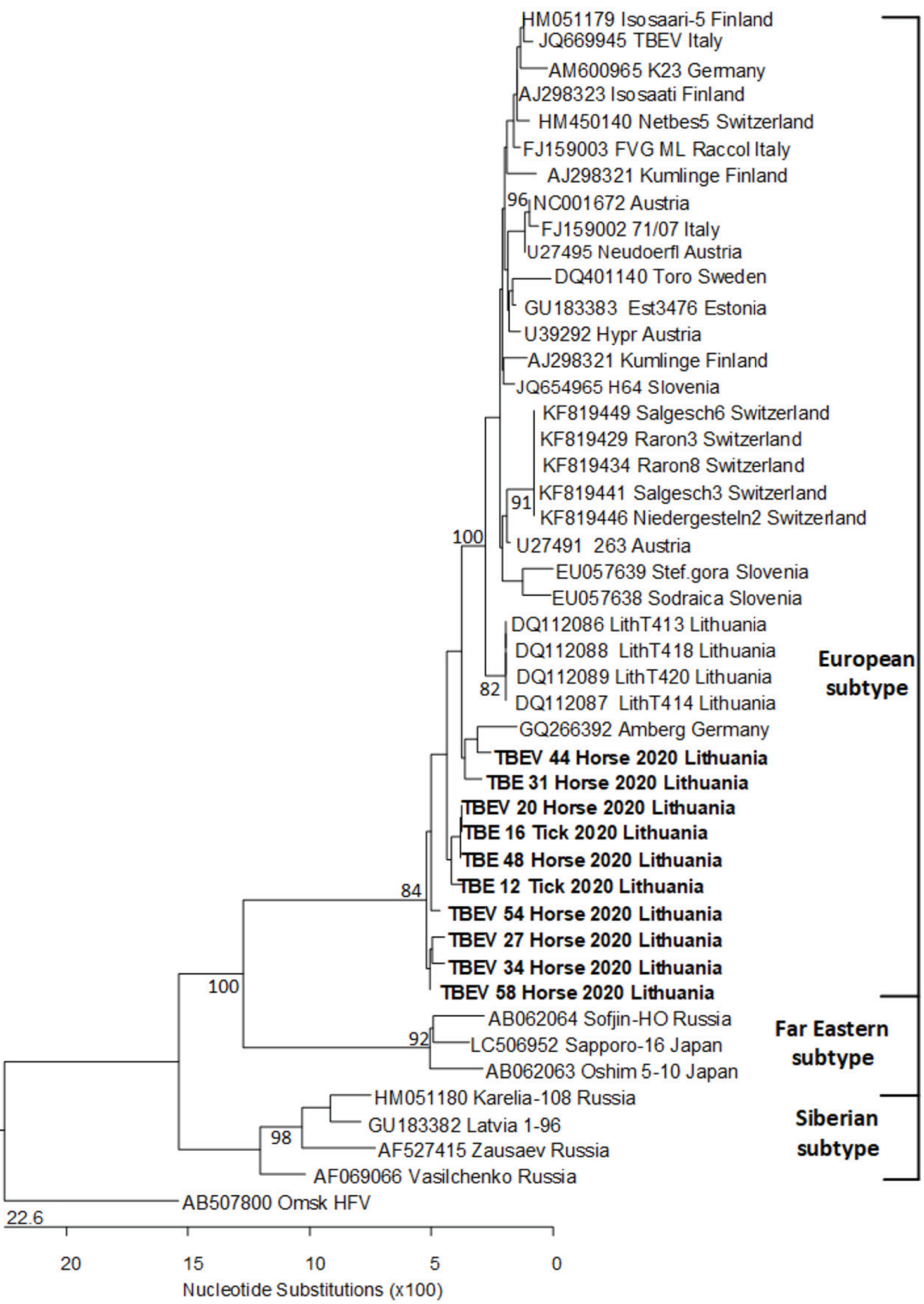


Fig. 3.3.2. *Phylogenetic tree of TBEV sequences obtained from horse serum and ticks collected from latter hosts.*

According to the results of the ELISA assay, 124 (41.2 %), 5 (1.7 %), and 172 (57.1 %) serum samples were positive, borderline, and negative for the presence of TBEV-specific antibodies, respectively. As a result, the optical density plot revealed a distinct bimodal distribution (Fig. 3.3.3.).

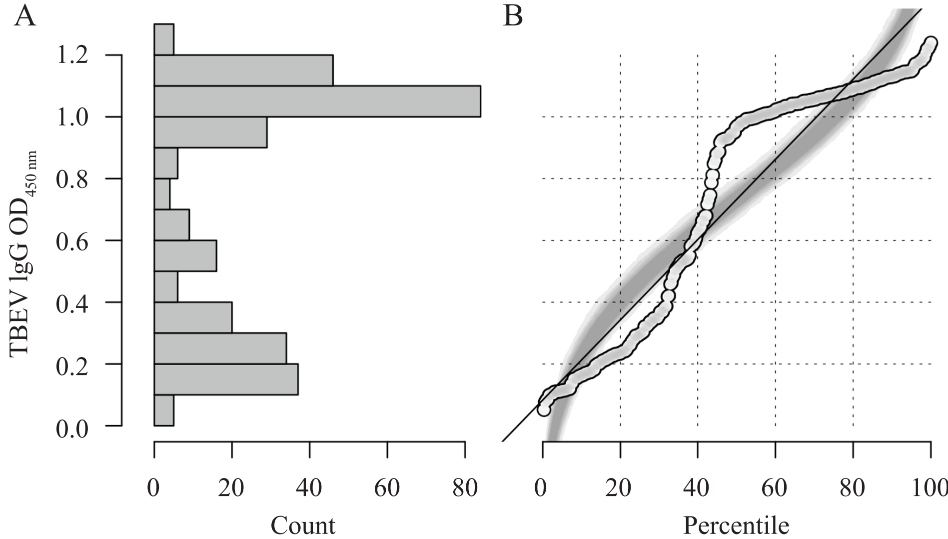


Fig. 3.3.3. *Normal quantile plot of the distribution of optical density (OD) 450 nm values in Tick-borne encephalitis virus (TBEV) immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) with sera collected from horses (A). Percentile plot with simulation (background) of a normal distribution with the same mean and standard deviation as for the data collected (B).*

The viral neutralization assay was used to retest all of the samples. ELISA negative samples were consistent with VNT. However, in the ELISA-positive and ELISA-borderline groups, only 109 and 4 samples were considered as positive, respectively. As a result, based on VNT results, the overall number of TBE seropositive samples was adjusted to 113 (37.5 %; 95 % CI 32.2–43.1).

The statistical associations between ration composition, herd size, age, gender, and seropositivity were not statistically significant. Contrary to tendency observed in Austria [31], we did not identify any significant relationships between the horse's sex. Different management conditions enforced on stallions in Austria may have influenced this result, whereas in our study, stallions were mainly kept in similar environment to those of mares. Furthermore, high tick density and infectivity in Lithuania could be a contributing factor in non-discriminating results in both sexes. However, binary logistic regression model showed significant association between pedigree and sero-

logical results. 31.0 % (95 % CI 24.9–37.6) of all tested samples were found to be positive in horses with known origin and 53.4 % (95 % CI 42.41–64.3) in mix-breeds ($p < 0.03$; OR 2.3; 95 % CI 1.3–4.0; β 0.8; SE 0.3). As much as it can be tempting to rely on genetics we believe that this association is solely due to the differences in management and purpose of mixed and purebred horses. Horses with no pedigree are more likely to be employed for lengthy walks in the nature, as well as for recreation and tourism. They were also kept in pastures for considerably longer periods of time, which boosters the odds that horses will encounter TBEV.

Furthermore, association between daily time spent in the pastures and seropositivity was observed. Horses spending >8 hours in the field had more than twice the seroprevalence (68.0 %; 95 % CI 59.8–75.4) than horses spending less than 8 hours daily (31.7 %; 95 % CI 18.0–48.0). Despite the evident differences, the final conclusion should be interpreted with caution as the data was gathered by a questionnaire survey of animal owners, which could be skewed by a bias.

There was no association between TBEV seropositivity and horse medical records. 11 horses of which 5 were tested positive for TBEV neutralizing antibodies demonstrated certain degree of atypical behavior or balance disturbances that was attributed to underdiagnosed cases of non-infectious origin.

One of the most significant results of this study was revealed by spatial analysis which showed statistically significant differences in mean seroprevalence amongst counties of the country. The highest seroprevalence rates were observed in east part of Lithuania with overall highest seroprevalence in Vilnius (50.6 %; 95 % CI 39.6–61.1) county ($p < 0.04$; OR 3.3; 95 % CI 1.0–10.7; β 1.2; SE 0.6). Moreover, strong correlation between TBEV seropositivity in tested horses and TBE-incidence rate in humans in given administrative unit was observed in 2018 ($r = 0.76$; $p < 0.05$) (Fig. 3.3.4. and Table 3.3.1.).

Interestingly, 11 equine serum samples appeared to be seropositive for West Nile virus and demonstrated cytopathic effect after VNT with TBEV, indicating first serological evidence of WNV in Lithuania. These findings call for more research into the possibility of a new WNV geographic distribution in northern Europe.

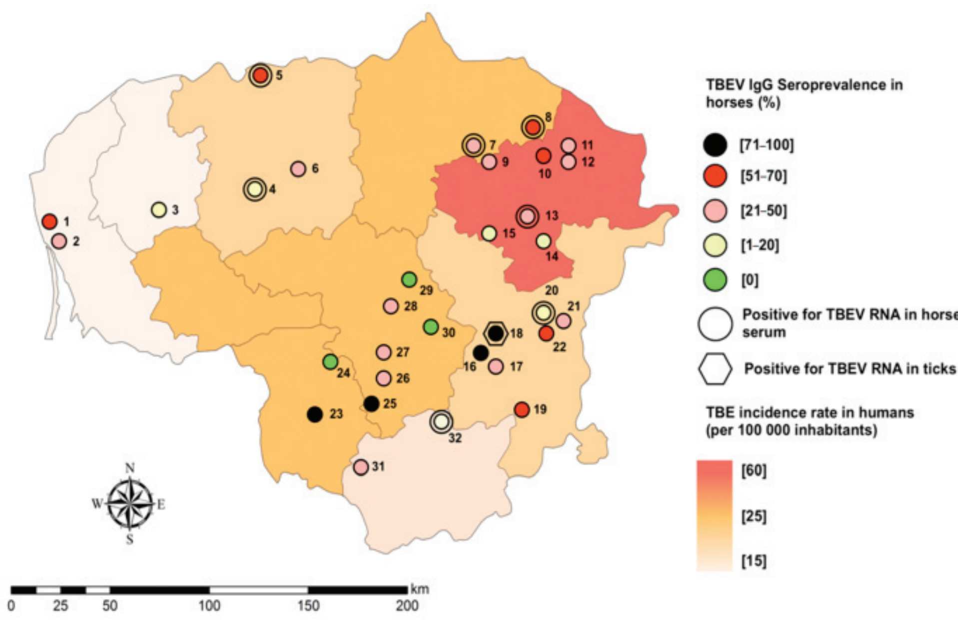


Fig. 3.3.4. *Spatial distribution of TBEV RNA and TBEV-neutralizing IgG seroprevalence at the NUTS3 level in Lithuania. The colour grades represent TBE incidence rate in humans.*

Table 3.3.1. *Results of equine serology in 32 tested stables. Stable number refers to the map in Fig. 3.3.3*

Stable number	Sample size of the Stable	County	Seropreva-lence in the County	TBEV Se-ropositive Horses	Seropreva-lence in the Stables	95 % CI
1	12	Klaipėda	42.3	8	66.6	34.8–90.0
2	14			3	21.4	0.4–50.8
3	12	Telsiai	8.3	1	8.3	0.2–38.4
4	7	Siauliai	30.6	4	57.1	18.4–90.1
5	10			1	10.0	0.2–44.5
6	19			6	31.5	12.5–56.5
7	12	Panevezys	46.2	5	41.6	15.1–72.3
8	14			7	51.0	23.0–76.9
9	13	Utena	30.8	4	30.7	0.9–61.4
10	3			1	33.3	0.8–90.5
11	4			1	25.0	0.6–80.5
12	11			4	36.3	10.9–69.2
13	15			5	33.3	11.8–61.6
14	6			1	16.6	0.4–64.1

Table 3.3.1 continuation

Stable number	Sample size of the Stable	County	Seropreva-lence in the County	TBEV Se-ropositive Horses	Seropreva-lence in the Stables	95 % CI
15	8	Vilnius	50.6	1	12.5	0.3–52.6
16	6			6	100.0	54.0–100
17	2			1	50.0	0.1–98.7
18	14			14	100.0	0.7–100
19	17			9	52.9	27.8–77.0
20	17			2	11.7	0.1–36.4
21	17			7	41.1	18.4–67.0
22	6			4	66.6	22.2–95.6
23	2	Marijam-pole	22.2	2	100.0	15.8–100.0
24	7			0	0.0	0–40.9
25	4	Kaunas	34.2	4	100.0	39.7–100.0
26	6			2	33.3	0.4–77.7
27	8			2	25.0	3.1–65.0
28	13			5	38.4	13.8–68.4
29	1			0	0.0	0.0–97.5
30	6			0	0.0	0.0–45.9
31	5	Alytus	20.0	2	40.0	0.5–85.3
32	10			1	10.0	0.2–44.5
Total	301			113	37.5	32.3–43.1

3.4. TBEV prevalence in ticks

A total of 2685 questing ticks corresponding to 886 adults, 1329 nymphs, and 88 larvae of *Ixodes ricinus*, and 382 *Dermacentor reticulatus* adults were collected in close proximity to the farms where milk samples were collected. All of these ticks accounted for 283 pools, 9 of which were found to be positive for TBEV-RNA. All positive samples were detected at the two sites in both years of the study.

Geographical distribution of positive samples is shown in Fig 3.1.4. The overall minimum infectious rate (MIR) was 0.34 % (95 % CI 0.15–0.64). Detailed summary of positive samples in questing ticks is presented in Table 3.4.1.

Table 3.4.1. *Summary of TBEV positive sample characteristics in ticks*

Location ID	**10**	**10**	**12**	**12**
Year	2018	2019	2018	2019
Dermacentor reticulatus		4/1/0	13/4/1	3/1/0
MIR % (95 % CI)			7.7 (0.2–36)	
Ixodes ricinus	13/3/1	27/5/1	30/6/0	34/6/2
MIR % (95 % CI)	7.7 (0.2–36)	3.7 (0–18.9)		5.8 (0.7–19.7)
Nymphs	71/4/2	40/2/1	192/6/1	24/2/0
MIR % (95 % CI)	2.8 (0.3–9.8)	2.5 (0.6–13.1)	0.5 (0-2.9)	
Larvae			22/1/0	
Total	84/7/3	71/7/2	257/17/2	61/9/2
MIR % (95 % CI)	3.5 (0.7–10.0)	2.8 (0.34–9.8)	0.8 (0–2.8)	3.2 (0.4–11.3)

Location ID refers to the map in Fig. 3.1.1 The sequence of three numbers is explained as follows: total tick sample size/number of pools/number of positive pools.

A total of 241 attached or moving ticks corresponding to 152 adults and 89 nymphs of *Ixodes ricinus* were collected from equine hosts. 4/38 of tested tick pools were positive for TBEV-RNA. The overall MIR was 1.2 % (95 % CI 0.3–3.6). All positive tick samples were in adult tick stage of *I. ricinus* obtained from the same stable.

Amplification of 16S rRNA was successful in all randomly selected pools, indicating that there were no false negative results caused by tick-derived products inhibiting the PCR test. Phylogenetic analysis showed that all detected TBEV strains belong to the European subtype and demonstrated no differences in diversity and distribution in the phylogenetic tree compared to sequences obtained from horse serum or milk. Distribution of strains are shown in phylogenetic analysis of the respective study in Figure 3.1.1 and Figure 3.3.1.

In a countrywide study conducted in Lithuania from 2017 to 2019, where about 9000 ticks were analyzed, a very comparable TBEV prevalence pattern in ticks was reported, as well as a virtually same overall MIR as in TBEV prevalence results in questing ticks presented here [191]. The latter investigation likewise revealed a patchy TBEV distribution, with many administrative units appearing to be virus-free. These findings support prior observations of TBEV patchiness in ticks and a discrepancy between the number of clinical TBE cases in humans and TBEV prevalence in ticks [22, 194, 195, 200, 201].

The results showed that both, the overall minimal infectious rate and virus load were higher in ticks collected from animals (Figure 3.4.1.). This is also consistent with previously published findings demonstrating that tick infectivity is higher when ticks are collected near or from domestic animals [30]. Almost 3-fold higher minimal infectious rate in ticks is most likely attributable with changes in dynamics of virus reproduction. It has been experimentally proven that intensive viral replication commences during feeding of ticks leading to a dramatic increase in virus load [202]. In contrast, this is not the case for questing ticks resulting in low concentration of TBEV which therefore in many cases could be below detection threshold. The results of virus isolation add weight to these statements. Of all positive pools, 3/9 of questing ticks and 4/4 of ticks collected from equine hosts were successfully isolated in cell culture. In short, increased MIR of ticks collected from horses roaming large, well-defined areas signifies an alternative to labor and time consuming field collection of ticks. However, more detailed studies are needed to find out if high-resolution spatiotemporal prevalence assesment is possible using this approach.

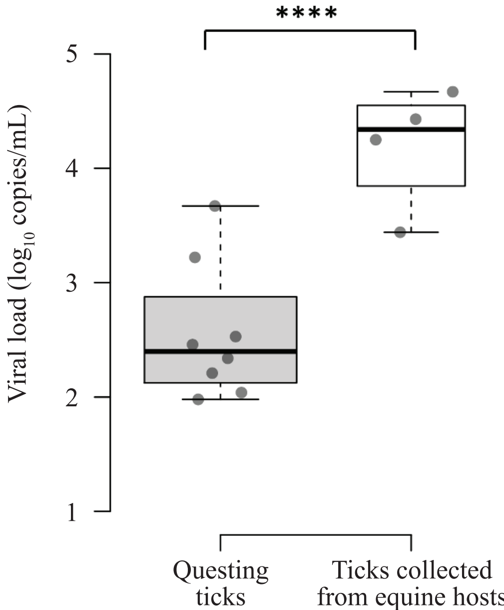


Fig. 3.4.1. *Viral load expressed as log_{10} viral RNA copies/mL.*

Asterisks and horizontal lines at the top of the graph indicate statistically significant differences (**** $p < 0.0001$).

3.5. Development of animal-based TBEV surveillance systems

The overall rationale of the research efforts presented here was not only investigate the TBEV prevalence in Lithuania, but also to analyze whether animal-based testing can be adopted as a TBEV surveillance strategy. Such efforts were stimulated by a fundamental basis of previously published results, indicating TBEV ecology is advantageous to animal-based surveillance systems. It has been amply shown that the TBEV prevalence in ticks removed from hosts are significantly higher compared to questing ticks [21, 202]. In addition, an experimental investigation found that TBEV replication is faster in feeding ticks, resulting in a 500-fold increase in viral load over a 15-hour observation period, but it is stable in unfed ticks [202]. Infected ticks were found to be the most active and aggressive, according to the same study. All this suggests that animals are likely to amplify the signal of TBEV presence in a given area, which in turn increases the chances of successful viral detection. As a result, an epidemiological monitoring strategy was conceptualized in which bulk milk tank samples were collected once every 4–5 days to cover the whole interval between possible infections, allowing to avoid missing the period when the virus is shed through milk. Longitudinal sample collection design was chosen based on earlier studies showing that TBEV is detectable in milk for 3–-8 days post infection [203] or slightly longer [40, 197]. As described earlier in paragraph 3.1. design shown no fundamental limitations as the number of positive samples could be overestimated only to a very limited level because of the possibility of misclassifying several samples of a single infection as separate cases.

TBEV surveillance by tick flagging was performed in parallel to the milk sample testing to validate results. It is important to sound a note that these techniques were different by their epidemiological design – tick flagging is cross-sectional study that typically involves collection of the samples once per season in particular site, while our proposed milk surveillance strategy is longitudinal, involving approximately 80 milk samples per season collected at regular 4–5 day intervals.

McNemar's test was used to determine the marginal homogeneity between two TBEV surveillance techniques. Ticks that were not collected on site due to their paucity were not included in the contingency table. Milk samples closest to tick samples in longitudinal scale were analyzed to assess the capacity of tick flagging and bulk milk testing strategies to identify TBEV prevalence. The data did not support the null hypothesis, implying that both approaches can identify TBEV when sampling time coincide.

Due to the differing study designs, straightforward statistical comparisons of the two testing approaches were not possible. Results, on the other hand,

revealed that both strategies may accurately assess the presence of the virus in the environment if they are used in close proximity. Thus, the flagging and dragging method could be considered as sensitive method for assessing TBEV prevalence geographically, but only if a large number of ticks are examined, which needs enormously high financial and people resources. This is a crucial point to emphasize in light of several studies that have failed to detect the virus in recognized TBE foci, despite testing tens of thousands of ticks [191, 194].

Given the very small sample size, milk analysis revealed the epidemiological situation in an incomparably higher resolution and demonstrated a number of advantages. Primarily, it was shown to be more reliable as it allowed the detection of TBEV circulation over a larger geographic area, resulting in nearly two-thirds of examined sites being positive, contrary to only two sites being positive for TBEV using the tick flagging method. The latter result echoes what other researchers have encountered – low TBEV prevalence rates and great spatiotemporal variability have been reported in a number of earlier investigations [23, 204–206]. Furthermore, the vast geographical distribution of TBEV in milk samples corresponded to the known TBE endemic area, and the monthly TBEV prevalence in milk samples was statistically associated with the monthly human TBE incidence rate. Milk analysis also proved to be more efficient in terms of both time and people resources. According to our estimates, collecting a single milk sample took no more than 5 minutes. Due to the small size of the country and well-developed road infrastructure, their periodic collection and transportation to the laboratory was similarly quick. Nonetheless, these findings are highly country-specific and should be interpreted as such. Finally, unlike the flagging and dragging method, the milk-based strategy poses no risk of being bitten, especially in the areas where Lyme borreliosis is prevalent and against which a vaccine does not yet exist.

It is important to emphasize that TBEV surveillance strategy based on milk sampling is in good agreement with CDC Guidelines for Evaluating Public Health Surveillance Systems recommendations [207]. In general, one of the important features of our proposed method is its **ease of use**. This strategy does not necessitate extensive collaboration with several institutions or highly qualified personnel. We also believe that this strategy is more efficient in terms of time, because sampling can be done by volunteer farmers, while milk collection may be ensured by specialists from local veterinary services on a regular basis. The **acceptability** requirement, i.e., everyone's willingness to use the procedure, was likewise met in full. The majority of the farmers graciously accepted to take part in the research project and expressed strong interest to be included as participants due to concerns about the quality and safety of their products. Paid testing may be considered if the engage-

ment was ongoing, especially as it would allow for a greater extension of the **flexibility** criterion. Using presently accessible infrastructure, milk testing for TBEV can be included into ongoing milk-borne disease monitoring programs or regular milk quality and safety management schemes. Despite the fact that our proposed strategy does not allow for **year-round monitoring**, the period when small ruminants graze and produce milk perfectly matches the seasonal distribution of confirmed TBE cases in humans [12]. Although the density of small ruminants appears to be significantly larger in Western Europe than in Lithuania [208], there seem to be some minor limitations in applying this strategy there due to non-homogenous geographic dispersion of farms. The bulk of small ruminant farms in Germany, for example, are located in the southern states [23]. In such cases, the strategy could be used at the very least to monitor endemic areas or to identify new TBEV foci.

Other aspects that comply with CDC criteria are **stability** and **availability.** Unpasteurized milk and it's products appear to be becoming more popular, owing to supposed health benefits and better taste [35]. The proprietors of the majority of the farms we visited consumed untreated milk products or produced them for sale. It is doubtful that this sector will lose its potential relevance to undertake a TBEV monitoring program as far as farm owners should adapt to market conditions.

Milk sample testing has the potential to provide an additional benefit: the assessment of milk safety. There have been few attempts to isolate TBEV from milk to date. Furthermore, rather than epidemiological screening, such efforts have primarily been used in investigations of alimentary outbreaks [154]. Although precise empirical data and extensive epidemiological research are lacking, it is estimated that milk is responsible for 7.8 % of TBE cases in Lithuania [12] and up to 17 % in Slovakia [157]. Our findings validated the importance of this issue, revealing that approximately one-fifth of positive milk samples were capable of infecting cells. Periodic milk testing and safety assessments for TBEV, as well as public education campaigns about the possible hazards of consuming untreated milk products, are critical in view of the high rates of alimentary transmission.

Many TBEV surveillance systems based on serological testing of diverse vertebrate hosts have recently been developed, showing promising results [27, 29, 89, 97, 99, 209]. Seroprevalence studies, however, have considerable drawbacks. As antibody persistence rates differ, it's difficult to evaluate when an infection occurred. Because of TBEV's well-known cross-reactivity with related flaviviruses [210], serological assessment necessitates confirmatory assays, which in turn, requires more labor and infrastructure.

Despite those limitations, our results proved that horses can be considered as possible sentinel specie for TBEV. It is known that horses are capable

to mount their Ab levels after contact with TBEV that could be detectable 9–19 months post-infection [30, 210, 211]. As this study revealed, interpreting seroprevalence at the stable level could allow mapping of TBEV foci on a smaller scale than district level, as high antibody values of positive horses at equestrian centers imply recent TBEV exposure.

Most of the criteria of CDC guideline are fully compliant with this approach. Exceptionally well-fulfilling criteria are year-round monitoring and flexibility as several countries have currently established routine monitoring programs for equine infectious anemia. Thus, large sample sizes are readily available and TBEV prevalence assessment can be integrated into ongoing studies. However, the criterion of acceptability and ease of use are only partially met, as this approach is interventional and requires not only qualified staff and bioethical authorization, but also consent from animal owners, often leading to many of them refusing to participate in this type of research due to adverse effects on animal productivity, sport results or well-being.

In short, analysis of bulk milk tank samples may serve as a valuable tool for TBEV prevalence assessment. As the technique is reliable, easy-to-operate and non-invasive it might be used for nationwide TBEV surveillance or be adapted to monitor endemic areas. In addition, TBEV surveillance studies in horses can reveal new microfoci and permit epidemiological mapping and therefore, complement human surveillance efforts.

CONCLUSIONS

1. TBEV-RNA was detected in 4.2 % (95 % CI 3.25–5.47) and 4.4 % (95 % CI 2.4–7.4) goat and sheep bulk milk tank samples, respectively. The overall MIR in questing ticks was only 0.3 % (CI 95 % 0.15–0.64). TBEV neutralizing antibodies (IgG) were found in 37.5 % (95 % CI 32.2–43.1) and TBEV-RNA were found in 3.9 % (95 % CI 2.3–6.8) horse serum samples. All detected TBEV-positive samples belonged to European subtype of TBEV.
2. Significantly more TBEV-positive milk samples were found in middle-size farms (6–20 animals) ($r = 0.54$, $p = 0.01$). The highest virus load was observed in small-size farms (1–5 animals) ($r = 0.64$, $p < 0.005$). 22.4 % (95 % CI 12.5–35.2) of positive samples were successfully isolated in MARC-145 and Vero cell cultures. Only 1 % (95 % CI 0.2–3) of all dairy products were positive for TBEV, demonstrating unsuccessful isolation in cell cultures and significantly lower virus load than in milk.

3.1. Milk sample testing has been found to be advantageous to tick flagging and dragging method because it allowed detection of TBEV-RNA 6.2 times more frequently in the geographical area; the prevalence was consistent with the incidence in humans ($r = 0.74$; $p = 0.035$) on a time scale and the geographical distribution coincided with the known TBE endemic area.

3.2. Serological testing of equine blood samples can be used for indirect assessment of the prevalence of TBEV, as it is geographically within the endemic range of EEV and coincides with human morbidity data ($r = 0.76$; $p < 0.05$).

SUMMARY IN LITHUANIAN

1. Problemos aktualumas ir svarba

Erkinis encefalitas (EE) yra svarbiausia erkių platinama virusinė zoonozinė liga Europoje, perduodama įsisiurbus erkinio encefalito viruso (EEV) užkrėstoms *Ixodes spp.* erkėms, kurios yra pagrindinis viruso pernešėjas ir rezervuaras [1, 2]. Alimentarinis užsikrėtimo kelias, kai užsikrečiama pavartojus nepasterizuoto pieno ar jo produktų yra žinomas kaip antrasis EE perdavimo būdas. Šiuo būdu dažniausiai užsikrečiama geriant ožkos pieną, tačiau yra užfiksuota atvejų, kai infekcija yra perduodama pavartojus ir karvių ar avių pieną [3–7]. Per 45 metų laikotarpį nuo 1970 iki 2014 metų, Lietuvoje sergamumas erkiniu encefalitu augo 8,5 procento per metus [12]. Nuo 2013 metų šios ligos sergamumo rodiklis yra didžiausias Europoje [9]. Be to, skirtingai nei kitose šalyse, erkinio encefalito užsikrėtimai fiksuojami visuose Lietuvos administraciniuose vienetuose, t.y. liga yra endeminė visoje Lietuvos teritorijoje [14].

Tikslinės ligos kontrolės priemonės turėtų būti diegiamos atsižvelgiant į detalius viruso paplitimo geografinėje erdvėje duomenis. Tačiau erkinio encefalito atveju, siekiant tai įgyvendinti, kyla kliūčių. Nors patvirtintų klinikinių erkinio encefalito atvejų skaičius su žinoma užsikrėtimo vieta gali būti tam tikru mastu pasitelkiamas regioninio lygmens EE rizikai nustatyti, pasikliauti vien žmonių sergamumo duomenimis negalima, nes nacionalinės sveikatos institucijos Europoje naudoja nevienodą rizikos vertinimo metodiką, diagnostikos procedūras bei ligos apibrėžimą [15]. Tokių duomenų naudojimas tiksliam viruso paplitimo įvertinimui tampa dar mažiau patikimu, jei EEV paplitimui įvertinti pasitelkiamos plačios geografinės teritorijos, nes erkių paplitimas yra nevienalytis ir neturi tiesioginio ryšio su žmonių populiacijos dydžiu konkrečioje geografinėje zonoje [16]. Tokio pobūdžio analizei įtakos gali turėti skirtingos vakcinacijos apimtys [17]. Duomenų tikslumui taip pat įtaką gali daryti ir socialiniai ir ekonominiai, politiniai, aplinkos ar klimato veiksniai [10, 18–20]. EEV cirkuliavimo geografinėje erdvėje vertinimui plačiausiai yra naudojamas būdas, kai yra renkamos ir erkinio encefalito atžvilgiu tiriamos erkės [21]. Tačiau šis metodas, besikaupiant vis daugiau mokslinių įrodymų, taip pat nebelaikomas patikimu, daugiausia dėl mažos teigiamų ėminių dalies bei geografinio ir temporalinio neatitikimo tarp viruso paplitimo erkėse ir žmonių sergamumo [22].

Pastaraisiais metais vis daugiau susidomėjimo kelia laukinių ir naminių gyvūnų tyrimai, siekiant netiesiogiai aptikti erkinio encefalito viruso cirkuliavimą [23, 24]. Žinomose endeminėse zonose buvo aptiktas statistiškai reikšmingas ryšys tarp erkiniam encefalitui specifinių antikūnų smulkių žinduolių,

šunų, galvijų ir elnių organizmuose bei žmonių sergamumo erkiniu encefalitu [25–29]. Tačiau tokio pobūdžio tyrimuose, arkliai, nors ir yra viena iš nedaugelio gyyūnų rūšių demonstruojančių klinikinius erkinio encefalito simptomus [30] sulaukė labai mažai dėmesio – atlikta tik keletas tyrimų kurių tikslas buvo įvertinti šios rūšies EE cirkuliavimo epidemiologinę reikšmę gamtoje [30–32].

Pieno mėginių tyrimai buvo įvardyti kaip dar vienas alternatyvus EE viruso paplitimo gamtoje vertinimo kelias [2]. Dviejų Švedijoje atliktų tyrimų rezultatai parodė, jog EEV specifinių antikūnų tyrimai gali pasitarnauti siekiant identifikuoti didesnės rizikos zonas [33, 34]. Vis dėlto, iki šiol buvo atlikti tik du tyrimai, kurių tikslas buvo ištirti erkinio encefalito viruso paplitimą piene. Abu tyrimai apėmė tik kiek daugiau nei šimtą mėginių, todėl dėl mažos imties, nebuvo padaryta situaciją sistemiškai apibrėžiančių išvadų [35, 36].

Taigi, pagrindinis šio tyrimo tikslas, buvo atlikti EEV paplitimo tyrimus mažiausiai tirtose srityse – arklių organizme ir naminių atrajotojų piene, bei įvertinti ar gauti rezultatai gali leisti sukurti viruso paplitimo gamtoje strategiją, savo ruožtu galinčią pakeisti arba papildyti dabartines viruso stebėsenos priemones.

2. Mokslinis naujumas

Erkinio encefalito viruso paplitimas naminių gyvūnų tarpe yra menkai tyrinėtas. Ypač mažai žinoma apie šio viruso paplitimą smulkiųjų atrajotojų piene – iki šiol buvo publikuotos tik dvi studijos, deja tyrusios kiek daugiau nei šimtą mėginių, todėl dėl imties ribotumo išsamių viruso paplitimo dėsningumų neatskleidusios. Šiame darbe aprašyti pirmieji išsamūs erkinio encefalito viruso paplitimo naminių ožkų ir avių piene rezultatai. Gauti duomenys atskleidė stebėtinai aukštą paplitimo mastą bei davė daug mokslui naujų žinių apie rizikos veiksnius, susijusius su pieno ir pieno produktų sauga. Duomenys taip pat paskatino sukurti naują, patikimą, neinvazinį ir paprastai naudojamą metodą, skirtą viruso cirkuliavimo gamtoje stebėsenai, kuris, tikimasi, gali pasitarnauti kaip nacionalinė erkinio encefalito stebėsenos strategija arba gali būti pritaikytas endeminėms vietovėms stebėti kitose šalyse. Šiame darbe aprašyti rezultatai taip pat apima išsamią erkinio encefalito virusui specifinių antikūnų paplitimo arkliuose analizę. Kartu yra aprašyti ir pirmieji sėkmingo erkinio encefalito viruso aptikimo arklių organizme bei sekoskaitos rezultatai. Atliekant tyrimus, buvo gauta ir kitų, nenumatytų, tačiau reikšmingų rezultatų. Buvo nustatyta, kad erkinio encefalito virusas gali būti izoliuotas MARC-145 persėjamojoje ląstelių linijoje. Kadangi šios ląstelės yra lengvai kultivuojamos ir nereiklios specifinėms sąlygoms, gautos žinios gali būti pritaikytos tyrimuose ateityje. Vykdant serologinius tyrimus buvo aptikti

pirmieji netiesioginiai įrodymai apie galimą Vakarų Nilo karštligės sukėlėjo cirkuliaciją Lietuvos teritorijoje.

3. Darbo tikslas ir uždaviniai

Darbo tikslas – nustatyti erkinio encefalito viruso paplitimą Lietuvos naminių gyvūnų populiacijose bei erkėse ir sukurti naują viruso paplitimo gamtoje stebėsenos sistemą.

Uždaviniai:

1. Įvertinti erkinio encefalito viruso paplitimą smulkiųjų atrajotojų, arklių ir erkių tarpe bei įvertinti jam įtaką darančius veiksnius
2. Įvertinti nepasterizuoto pieno ir jo produktų saugą.
3. Sukurti erkinio encefalito viruso paplitimo gamtoje stebėsenos sistemą ir įvertinti jos efektyvumą.

4. Tyrimo metodai ir tiriamoji medžiaga

4.1. Arklių serumo mėginiai

Remiantis daugiasluoksne atsitiktinių imčių mėginių rinkimo strategija ir siekiant surinkti reprezentatyvią, Lietuvoje esančią situaciją atspindinčią imtį, iš skirtingose Lietuvos apskrityse laikomų arklių buvo surinktas 301 serumo mėginys. Mėginių skaičius iš kiekvienos apskrities buvo surinktas atsižvelgiant į toje apskrityje laikomų arklių skaičių su 5 proc. paklaida (95 proc. PI). Formuojant imtį buvo remiamasi Nacionalinės žemės tarnybos prie Lietuvos Respublikos žemės ūkio ministerijos duomenų bazės duomeninis. Kiekvienoje vietoje mėginiai buvo renkami vieną kartą. Gyvūnai buvo įtraukti į tyrimą jei atitiko sekančius kriterijus: turėjo aiškiai apibrėžtą ganyklų plotą, kiekvieną dieną buvo ganomi lauke, nebuvo vakcinuoti prieš flavivirusų sukeliamas infekcijas.

4.2. Ožkų ir avių pieno mėginiai

Remiantis aukščiau aprašyta mėginių rinkimo strategija, buvo surinkti 1363 ožkų ir 312 avių pieno mėginiai. Dėl žinomo trumpo EEV viremijos laikotarpio, pieno mėginiai buvo imami periodiškai kartą kas 4–5 dienas iš bendros pamelžto pieno talpos. Mėginiai buvo renkami visą laktacijos ir erkių aktyvumo laikotarpį trunkantį nuo balandžio iki lapkričio mėnesio. Mėginiai buvo renkami du sezonus – 2018 ir 2019 m.

Formuojant imtį buvo remiamasi Lietuvos nacionalinio ūkinių gyvūnų registro duomenų bazės duomenimis. Gyvūnai buvo įtraukti į tyrimą jei atitiko

šiuos kriterijus: ožkos ir avys nebuvo vakcinuotos nuo flavivirusų sukeliamų infekcijų, joms netaikytos prevencinės priemonės prieš erkes, ūkiai buvo įsikūrę ne miesto teritorijoje ir savo teritorijoje netaikė erkių kontrolės priemonių. Mėginiai buvo paimti iš įvairaus dydžio ūkių (mažų – 1–5 melžiami gyvūnai; vidutinio dydžio – 6–20 ir didelių – ≥21).

4.3. Nepasterizuoto pieno produktų mėginiai

Siekiant įvertinti nepasterizuotų pieno produktų saugą erkinio encefalito atžvilgiu, buvo surinkti 247 sūriai (švieži, rūgštiniai sūriai, n = 82; 30 dienų brandinimo sūris (± 5 dienos), n = 81; >60 dienų brandinimo sūriai, n = 84) ir 42 jogurto mėginiai. Iš kiekvieno pieno produkto buvo suformuotas 300 mg mėginys tolimesniems tyrimams. Visi mėginiai buvo gauti tiesiogiai iš ūkininkų arba įsigyti turguose. Mėginiai atspindi 18 skirtingų gamintojų produkciją. Kadangi šie mėginiai buvo skirti tik produktų saugumui įvertinti, o ne erkinio encefalito viruso geografiniam paplitimui tirti, geografinės koordinatės, atspindinčios gamybos vietą, nedetalizuojamos.

4.4. Erkės

Iš viso buvo surinktos 2925 erkės. 2684 erkės buvo surinktos ūkių, kuriuose buvo renkami pieno mėginiai apylinkėse. Likusios 241 erkės buvo surinktos nuo arklių kūnų. Kaip įprasta tokio pobūdžio tyrimuose, erkės buvo renkamos vieną kartą per sezoną.

Erkių jungtiniai mėginiai buvo suformuoti priklausomai nuo vystymosi stadijos, lyties, rūšies ir jų surinkimo vietos. Kiekvieną jungtinį mėginį sudarė iki 10 *Ixodes ricinus* suaugusiųjų, 20 nimfų, 50 lervų arba 5 *Dermacentor reticulatus* erkių. Surinkus, erkės buvo gyvos transportuojamos į laboratoriją, kur buvo atliekamas erkių homogenizavimas paveikus skystu azotu ir suspensijų fosfatiniame buferyje ruošimas.

4.5. Imunofermentinė tyrimų analizė

Arklių serumo mėginiai siekiant nustatyti EEV specifinius G klasės antikūnus buvo ištirti ELISA imunofermentinės analizės metodu, naudojant EIA TBEV IgG rinkinį (TestLine Clinical Diagnostics, Čekija) remiantis gamintojo instrukcijomis.

Siekiant išvengti galimo kryžminio reaktyvumo su kitais flavivirusais, visi arklių serumo mėginiai buvo pakartotinai ištirti taikant virusų neutralizacijos testą (VNT). Prieš atliekant tyrimą, visuose mėginiuose buvo pašalinti komplemento baltymai. Po to mėginiai buvo praskiesti nuo 1/5 iki 1/320 mitybinėje terpėje (MEM). Praskiesti serumai buvo inkubuojami su 100 $TCID_{50}$

EEV padermės, gautos po 6 auginimo ciklų Vero ląstelių kultūroje (ATCC® CCL-81™). Praėjus 3, 5 ir 7 dienoms po užsikrėtimo buvo vertinamas citopatinis poveikis.

Mėginiai buvo pakartotinai ištirti ELISA testu, siekiant nustatyti G klasės antikūnus prieš Vakarų Nilo virusą, naudojant WN Competition Multispecies Ig rinkinį (ID.vet, Prancūzija).

4.6. EEV viruso aptikimas ir sekoskaita

Virusinė RNR iš 300 µl nugriebto pieno, centrifuguotų erkių arba ląstelių supernatanto arba serumo buvo išskirta naudojant GeneJET RNA išskyrimo rinkinį (Thermo Scientific, JAV) remiantis gamintojo instrukcijomis. Mėginiai buvo tiriami tikro laiko PGR ir konvenciniu PGR, naudojant pradmenų rinkinius pateiktus 2.6.1 lentelėje.

Lentelė 2.6.1. *Oligonukleotidiniai pradmenys naudoti tyrime*

Pradmuo/zondas	Pradmenų seka (5’ to 3’)	Šaltinis
F-TBE 1	GGGCGGTTCTTCTCC	[183]
R-TBE 1	ACACATCACCACCTCCTTGTCAGACT	
TBE-WT	FAM-TGAGCCACCATCACCCAGACACA-TAMRA	
TBE NCR5F	GCGTTTGCTTCGGA	[184]
TBE NCR5R	CTCTTTCGACACTCGTCGAGG	
TBE NCR5Fn	CGGATAGCATTAGCAGCG	
TBE NCR5Rn	CCTTTCAGGATGGCCTT	
TBE NS5F	GAGGCTGAACAACTGCACG	[185]
TBE NS5R	GAACACGTCCATTCCTGATCT	
TBE NS5Fn	ACGGAACGTGACAAGGCTAG	
TBE NS5Rn	GCTTGTTACCATCTTTGGAG	
F-16s-Ixodes	AAAAAAATACTCTAGGGATAACAGCGTAA	[183]
R-16s-Ixodes	ACCAAAAAAGAATCCTAATCCAACA	
16s-Ixodes-Probe	TTTTGGATAGTTCATATAGATAAAATAGTTTGC GACCTCG	

Tikro laiko polimerazinei grandininei reakcijai buvo naudojama SuperScript III Platinum One-Step Taq Mix atvirkštinė transkriptazė ir 2x Reaction Mix reakcijos mišinys (Thermo Scientific, JAV). Lizdinei polimerazinei grandininei reakcijai – DreamTaq Green PCR Master Mix (2x) reakcijos mišinys, RevertAid atvirkštinė transkriptazė (200 U/µl) ir RiboLock RNazės inhibitorius (40 U/µl). Likę reagentai ir temperatūriniai režimai taikyti atsižvelgiant į originalias tyrimo metodiką aprašiusias publikacijas. RNA išskyrimo efektyvumo ir kokybės kontrolė buvo įvykdyta taip kaip aprašyta anks-

čiau [183]. Visų teigiamų mėginių specifiškumas buvo patvirtintas NCR5 arba NS5 viruso genomo fragmento sekoskaita.

4.7. Viruso kopijų skaičiaus apskaičiavimas

Viruso genomo NS5 fragmentas buvo klonuotas į pJET1.2 vektorių, naudojant CloneJET PCR klonavimo rinkinį (Thermo Scientific, JAV) ir Transform-Aid Bacterial Transformation Kit (Thermo Scientific, JAV) rinkinį, remiantis gamintojo nurodymais. Plazmidės DNR išskyrimas ir gryninimas buvo atliktas naudojant GeneJET Plasmid Miniprep rinkinį (Thermo Scientific, JAV) ir kiekybiškai įvertintas naudojant Qubit dsDNA BR Assay Kit (Invitrogen) pagal gamintojo nurodymus. Standartinės kreivės buvo sukurtos po 10 kartų praskiedus pradinę DNR. Pradinė mėginio koncentracija buvo išreikšta fluorescencijos vienetais ir konvertuota į genomo kopijų skaičių/ml. Kiekybinis viruso kopijų nustatymas buvo atliekamas taikant tikro laiko PGR metodą su SYBR Green I dažais (Thermo Scientific, JAV), NS5 pradmenimis, atitinkamais, aukščiau aprašytais temperatūriniais režimais.

4.8. Viruso izoliavimas ląstelių kultūrose

Siekiant įvertinti gebėjimą užkrėsti ląsteles, teigiami mėginiai buvo izoliuoti Vero (ATCC® CCL-81™) ir MARC-145 (ATCC® CCL-12231™) ląstelių linijose. Ląstelės buvo kultivuojamos 37 °C temperatūroje 5 proc. CO_2 ir oro mišinyje, mitybinėje terpėje (MEM, Gibco, JAV), turinčioje 10 proc. termiškai inaktyvuoto galvijų vaisiaus serumo (FBS; Gibco, JAV) bei 100 U ml^{-1} penicilino ir 100 µg l^{-1} streptomicino.

Atrinkti teigiami mėginiai buvo filtruojami per 0,22 µm mikrofiltrą (Techno Plastic Products AG, Šveicarija). Ląstelės buvo inokuliuojamos 25 cm^2 plastikinėse plokštelėse (TPP Techno Plastic Products AG, Šveicarija) 1 valandą 37 °C temperatūroje. Vėliau buvo stebimas citopatinio efekto pasireiškimas atliekant 6 ląstelių persėjimo ciklus.

4.9. Statistinė analizė

Pasikliautiniesiems intervalams apskaičiuoti buvo naudojamas binominis metodas. Rizikos veiksnių reikšmingumui patikrinti buvo naudojama dvejetainė logistinės regresijos analizė, šansų santykis (OR), regresijos koeficientas (β), standartinė paklaida (SE) ir chi kvadrato testas. Pearsono koreliacija buvo naudojama siekiant įvertinti žmonių ir gyvūnų EEV paplitimo ryšį. Minimalus užsikrėtimo dažnis (MIR) buvo apskaičiuotas kaip teigiamų jungtinių mėginių skaičiaus ir bendro ištirtų erkių skaičiaus santykis. MIR = teigiamų jungtinių mėginių skaičius / bendras tirtų erkių skaičius × 100 proc.

Dichotominio priklausomo kintamojo skirtumai tarp dviejų susijusių grupių buvo įvertinti MnNemaro testu. Statistinė analizė ir vizualizacijos buvo atlikti naudojant programavimo kalbą R.

4.10. Finansavimas

Tyrimas finansuotas Lietuvos Respublikos žemės ūkio ministerijos projekto Nr. MT-18-5. Tyrimai iš dalies paremti Lietuvos sveikatos mokslų universiteto Atviro fondo dotacijomis.

5. Rezultatai

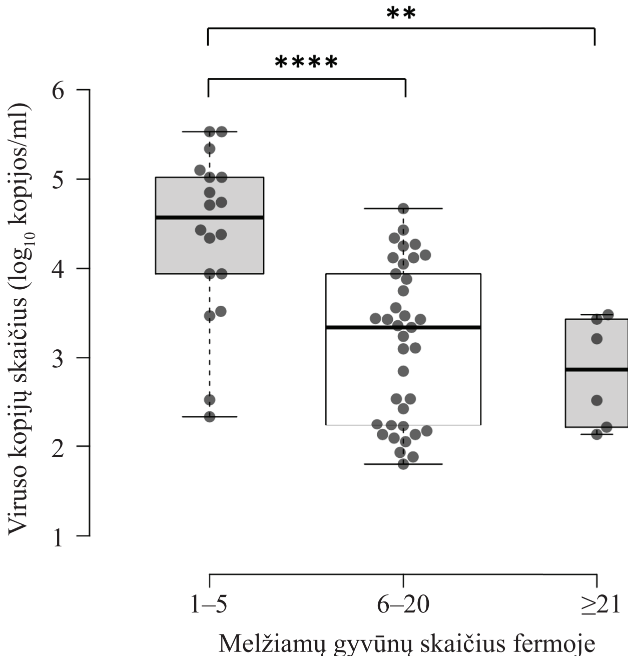
5.1. EEV paplitimas ožkų ir avių piene

Atitinkamai 4,3 proc. (95 proc. PI 3,3–5,5) ir 4,5 proc. (95 proc. PI 2,5–7,4) ožkų ir avių pieno mėginių buvo teigiami EEV RNR atžvilgiu. Visų teigiamų mėginių specifiškumas buvo patvirtintas daliniu genomo sekos nustatymu. Sekos patalpintos į Genbank duomenų bazę (sekų prieigos nr.: MZ664211-MZ664256). BLASTN paieška (NCBI Genbank) atskleidė, kad visos sekos, gautos iš pieno mėginių, buvo 89,2–94,1 proc. panašios į Europos TBEV potipio (Neudörfl; Genbank U27495) etaloninę padermę.

2018 ir 2019 metais bent vienas teigiamas mėginys buvo nustatytas atitinkamai 70,6 proc. ir 64,7 proc. tirtų ožkų ūkių. 75 proc. avių ūkių bent vienas teigiamas mėginys buvo nustatytas abejais tyrimų metais. Nebuvo statistiškai reikšmingų skirtumų tarp rūšių nė viename iš tirtų rizikos veiksnių.

Rezultatai parodė, kad EEV teigiamų mėginių kiekis varijuoja laiko skalėje ir tarp abejų tyrimo metų, tačiau statistiškai reikšmingų skirtumų neaptikta. Nepaisant to, analizė atskleidė ryšį tarp viruso paplitimo tarp gyvūnų ir sergamumo erkiniu encefalitu žmonių tarpe 2019 m. ($r = 0{,}74$; $p = 0{,}035$). Deja, 2018 m. ta pati priklausomybė ($r = 0{,}65$) nebuvo statistiškai reikšminga, nors demonstravo tą pačią tendenciją ($p = 0{,}083$). Erdvinė analizė parodė, kad teigiami mėginiai buvo tolygiai pasiskirstę visoje Lietuvos teritorijoje.

Buvo nustatyta, kad ūkio dydis (6–20 > 1–5 > daugiau nei 21 gyvūnas) ir teigiamų mėginių skaičius koreliavo tarpusavyje ($r = 0{,}54$, $p = 0{,}01$). Be to, buvo nustatytas ir tiesinis ryšys tarp ūkyje pagaminamo pieno kiekio ir jame aptinkamo virusinių dalelių skaičiaus ($r = 0{,}64$, $p < 0{,}005$).



Pav. 5.1.1. *Virusinių kopijų skaičius išreikštas log_{10} RNR kopijos/ml*
(** $p < 0{,}01$; **** $p < 0{,}0001$).

22,4 proc. (95 proc. PI 12,5–35,2) ožkų pieno mėginių, kuriuose buvo aptiktas EEV pasižymėjo gebėjimu infekuoti ląsteles ir sukėlė citopatinį efektą praėjus 4-6 dienoms po užsikrėtimo. Nors citopatinio poveikio išraiška ir viruso kopijų skaičius variavo, rezultatai tarp Vero ir MARC-145 ląstelių linijų reikšmingai nesiskyrė. Mūsų žiniomis, tai yra pirmasis tyrimas atskleidžiantis, jog erkinio encefalito virusas gali būti izoliuojamas ir gausinamas MARC-145 ląstelių linijoje, o tai savo ruožtu yra reikšmingas turimų žinių apie viruso savybes papildymas, ateityje leisiantis virusą izoliuoti šioje ypač lengvai kultivuojamoje ir specifinėms sąlygoms nereiklioje ląstelių linijoje.

5.2. EEV aptikimas pieno produktuose

Iš 247 skirtingo brandinimo laipsnio sūrio ir 42 jogurto mėginių tik trijuose buvo aptiktas erkinio encefalito virusas. Visi trys teigiami mėginiai buvo aptikti šviežiuose rūgštiniuose sūryje. Teigiami mėginiai sudarė 1 proc. (95 proc. PI 0,2–3,0) visų tirtų pieno produktų. Siekiant patvirtinti TBEV specifiškumą, buvo atlikta dalinė viruso genomo sekoskaita. Filogenetinė analizė parodė, kad visos aptiktos TBEV padermės priklauso Europos potipiui. Nė vienas iš teigiamų mėginių nebuvo išskirtas ląstelių kultūroje. Viruso kopijų

skaičius aptiktuose mėginiuose siekė 2,1 log_{10} ir buvo statistiškai reikšmingai mažesnis nei pieno mėginiuose.

Nors ir trūksta eksperimentinių rezultatų, leidžiančių tiksliai apibūdinti esamą situaciją, galima spėti, kad ilgiau brandintuose sūriuose virusas ilgainiui praranda savo struktūrą ir tampa neužkrečiamas dėl ilgesnio temperatūros ir UV spindulių poveikio. Taigi, produktai, kurių technologinis procesas yra ilgesnis, tikėtina yra gerokai saugesni erkinio encefalito atžvilgiu.

5.3. EEV aptikimas arklių kraujo serume

EEV virusas buvo rastas 3,9 proc. (95 proc. PI 2,3–6,8) iš visų tirtų arklių kraujo serumo mėginių. Remiantis BLASTN paieška (NCBI Genbank), visos iš arklių serumo gautos sekos buvo 93,0–97,1 proc. identiškos Europos potipio (Neudörfl; Genbank U27495) etaloninei padermei. Panašumas su Tolimųjų Rytų potipio (Sofjin; AB062064) ir Sibiro potipio (Vasilchenko; AF069066) etaloninėmis padermėmis buvo atitinkamai 78,3 proc. ir 84,4 proc.. Sekos buvo patalpintos į GenBank duomenų bazę (sekų prieigos nr.: MT981174-MT981178 ir MW187721-MW187725). Dėl trumpo viremijos laikotarpio, po kurio flavivirusai yra pašalinami iš kraujo, tiesioginis erkinio encefalito viruso aptikimas stambiuose žolėdžiuose yra ribotas. Mūsų žiniomis, šiame darbe aptarti rezultatai apie tiesioginį viruso aptikimą arklių serume yra publikuojami pirmą kartą.

Atlikus imunofermentinę analizę ir aptiktus teigiamus mėginius pakartotinai ištyrus taikant viruso neutralizacijos reakciją, nustatyta, jog specifinių neutralizuojančių G klasės antikūnų turėjo 37,5 proc.; (95 proc. PI 32,2–43,1) tirtų mėginių. Ryšiai tarp raciono sudėties, bandos dydžio, amžiaus, lyties ir serologiškai teigiamų mėginių skaičiaus nebuvo statistiškai reikšmingi. Tačiau buvo aptikti reikšmingi skirtumai tarp kasdienio ganyklose praleisto laiko ir serologiškai teigiamų mėginių skaičiaus. Arklių, kurie ganomi ilgiau nei 8 valandas, organizme EEV-specifiniai antikūnai buvo aptinkami du kartus dažniau (68,0 proc.; 95 proc. PI 59,8–75,4) nei arkliuose besiganančiuose trumpesnį laiką (31,7 proc.; 95 proc. PI 18,0–48,0).

Logistinės regresijos modelis parodė reikšmingą ryšį tarp arklių kilmės ir serologinių rezultatų. Iš visų serologiškai tirtų mėginių, 31,0 proc. (95 proc. PI 24,9–37,6) buvo teigiami grynaveislių arklių tarpe ir 53,4 proc. (95 proc. PI 42,4–64,3) - mišrių veislių ($p < 0{,}03$; OR 2,3; 95 proc. PI 1,3–4,0; β 0,8; SE 0,3). Išanalizavus metaduomenis paaiškėjo, kad šie skirtumai yra susiję ne su genetiniais veiksniais, bet arklių paskirties ypatybėmis. Į šį tyrimą įtraukti negrynaveisliai arkliai daugiau laiko praleisdavo gamtoje ir buvo dažniau naudojami ilgiems žygiams, o tai savo ruožtu didino erkių įsisiurbimo tikimybę. Nors arkliai yra viena iš nedaugelio rūšių, kuriems būdingi klinikiniai

erkinio encefalito simptomai, nebuvo aptikta jokio ryšio tarp serologiškai teigiamų mėginių skaičiaus ir arklių ligos istorijų.

Vieną reikšmingiausių šio tyrimo rezultatų atskleidė erdvinė analizė, kuri parodė statistiškai reikšmingus serologiškai teigiamų mėginių pasiskirstymo skirtumus tarp šalies apskričių. Didžiausias serologinio paplitimo rodiklis buvo rytinėje Lietuvos dalyje, o bendras didžiausias serologinis paplitimas Vilniaus apskrityje (50,6 proc.; 95 proc. PI. 39,6–61,1) ($p < 0,04$; OR 3,3; 95 proc. PI 1,0–10,7; β 1,2; SE 0,6). Negana to, nustatyta stipri koreliacija tarp serologiškai teigiamų mėginių skaičiaus ir žmonių sergamumo erkiniu encefalitu geografinėje erdvėje ($r = 0,76$; $p < 0,05$).

Vienuolika arklių serumo mėginių buvo serologiškai teigiami Vakarų Nilo virusui ir demonstravo citopatinį poveikį po viruso neutralizacijos testo su EEV. Tai rodo pirmuosius netiesioginius Vakarų Nilo karštinės viruso cirkuliavimo Lietuvoje įrodymus.

5.4. EEV paplitimas erkėse

Tarp 2685 erkių, surinktų šalia ūkių, kuriuose buvo renkami pieno mėginiai, buvo identifikuotos 886 *Ixodes ricinus* rūšies suaugusios, 1329 nimfos ir 88 lervos stadijos erkės bei 382 *Dermacentor reticulatus* suaugusios erkės. Iš šių erkių buvo suformuoti 283 jungtiniai mėginiai, iš kurių devyniuose buvo aptikta EEV-RNR. Visi teigiami mėginiai buvo aptikti tik dviejose vietose. Bendras minimalus užkrėtimo dažnis erkėse (MIR) buvo 0,34 proc. (95 proc. PI 0,15–0,64).

Tarp 241 erkės surinktos nuo arklių kūnų buvo identifikuotos 152 suaugusios *Ixodes ricinus* erkės ir 89 nimfos. 4 iš 38 suformuotų jungtinių erkių mėginių buvo teigiami EEV-RNR atžvilgiu. Bendras MIR buvo 1,2 proc. (95 proc. PI 0,3–3,6). Visi teigiami erkių mėginiai buvo suaugusių *I. ricinus* erkių stadijoje, gauti iš tos pačios arklių laikymo vietos.

16S rRNR amplifikacija buvo sėkminga visuose atsitiktinai atrinktuose jungtiniuose mėginiuose, o tai rodo, kad nebuvo klaidingai neigiamų rezultatų, kuriuos galėjo sukelti erkėse aptinkamos medžiagos. Filogenetinė analizė parodė, kad visi aptikti EEV teigiami mėginiai priklauso Europos potipiui.

3/9 jungtiniai erkių, surinktų gamtoje, mėginiai ir 4/4 jungtiniai erkių, surinktų nuo arklių kūnų, mėginiai gebėjo infekuoti ląsteles. Erkėse surinktose nuo arklių taip pat buvo aptiktas statistiškai reikšmingai didesnis virusinių kopijų skaičius. Tai rodo, jog erkės surinktos nuo stambių žinduolių turi daugiau potencialo būti pasirinktos tyrimo objektu siekiant nustatyti erkinio encefalito paplitimą. Tačiau siekiant išsiaiškinti ar toks erkių rinkimas galėtų leisti tiksliai įvertinti viruso paplitimą laike ir erdvėje, reikia daugiau tyrimų.

5.5. Naujų EEV stebėsenos metodų plėtojimas

Bendras šio darbo siekis buvo ne tik ištirti EEV paplitimą Lietuvoje, bet ir išanalizuoti, ar tokio pobūdžio tyrimai galėtų pasitarnauti erkinio encefalito paplitimo gamtoje stebėsenos priemonių kūrimui.

Siekiant įgyvendinti šią idėją, buvo pasirinkta epidemiologinė mėginių rinkimo strategija, apimanti bendros pieno talpos mėginių rinkimą kas 4–-5 dienas, siekiant išvengti trumpos viruso viremijos sąlygoto periodo kai virusas nustoja būti išskirtas į pieną. Rezultatų palyginimui ir įvertinimui, erkės buvo renkamos ūkių, kuriuose buvo renkami pieno mėginiai, apylinkėse. Svarbu pažymėti, kad šie metodai skyrėsi savo epidemiologine struktūra – erkės buvo renkamos kiekvienoje vietoje tik vieną kartą per sezoną, kaip tai įprasta tokio pobūdžio tyrimuose, t.y. naudojama vienmomenčio skerspjūvio tyrimo strategija (angl. *cross-sectional study*). Pieno mėginių rinkimas buvo atliekamas ilgalaikio tyrimo pagrindu (angl. *longitudinal study*) apimantis maždaug 80 mėginių per sezoną renkamų reguliariais intervalais.

Ribiniam homogeniškumui nustatyti tarp dviejų tyrimo strategijų buvo naudojamas McNemaro testas. Į modelį buvo įtraukti pieno mėginiai, kurių ėmimo laikas sutapo ar buvo artimiausias konkrečios tyrimo vietos erkių surinkimo laikui. Gauti rezultatai nepatvirtino nulinės hipotezės, o tai reiškia, kad abu metodai geba vienodai gerai identifikuoti EEV, kai mėginių ėmimo laikas sutampa.

Vis dėlto, taikytų epidemiologinių tyrimo struktūrų skirtumai parodė pieno tyrimų pranašumą. Atsižvelgiant į sąlyginai mažą imties dydį, pieno mėginių analizė atskleidė epidemiologinę situaciją nepalyginamai detaliau. Ši strategija leido aptikti EEV cirkuliavimą didesniame geografiniame plote – 70,6 proc. tirtų vietų buvo teigiamos EEV atžvilgiu, kai tiriant erkes virusas buvo aptiktas tik 11,4 proc. tirtų vietų. Be to, platus EEV geografinis pasiskirstymas pieno mėginiuose atitiko žinomos endeminės erkinio encefalito teritorijos ribas, o EEV paplitimas pieno mėginiuose laiko skalėje buvo statistiškai susijęs su mėnesiniu žmonių sergamumo EE dažniu.

Pažymėtina, kad pieno mėginių tyrimų strategija turi ir kitų pranašumų. Ji yra neinvazinė, todėl nedaro žalos gyvūnams ir nemažina jų produktyvumo. Taip pat nereikalauja bioetikos leidimų. Be to, pieno mėginių tyrimai yra saugūs, priešingai nei tyrimai, kai yra renkamos erkės. Šiuo atveju egzistuoja didelė erkių įsisiurbimo ir ligų sukėlėjų perdavimo tikimybė.

Svarbu pabrėžti, kad EEV stebėjimo strategija, pagrįsta pieno mėginių ėmimu, atitinka visas ligų kontrolės ir prevencijos centro suformuotas visuomenės sveikatos priežiūros sistemų vertinimo rekomendacijas: paprastumo kriterijų (nesudėtingas mėginių rinkimas, nereikalingas ypač aukštos kvalifikacijos personalas), lankstumo kriterijus (tyrimai gali būti vykdomi kaip

atskira programa arba būti įtraukti į egzistuojančias per pieną plintančių ligų stebėsenos programas arba į reguliarias pieno kokybės ir saugos valdymo programas), stebėsenos ištisus metus kriterijus (erkių aktyvumo ir smulkiųjų atrajotojų ganiavos periodas sutampa), stabilumo kriterijus (nepasterizuoto pieno ir jo produktų vartojimas auga). Kaip aprašyta ankščiau, ši stebėsenos strategija, kartu leidžia įvertinti ir pieno saugumą erkinio encefalito atžvilgiu.

Arklių molekuliniai ir serologiniai tyrimai parodė, kad ši rūšis gali pasitarnauti siekiant įvertinti erkinio encefalito viruso paplitimą. Erdvinė analizė parodė, jog serologinio paplitimo duomenys detaliai atskleidžia viruso geografinę cirkuliavimo teritoriją bei sutampa su žmonių sergamumo duomenimis. Pažymėtina, jog tokio pobūdžio tyrimai atitinka visuomenės sveikatos priežiūros sistemų vertinimo rekomendacijas. Ypač pabrėžtinas lankstumo kriterijus, nes EEV paplitimo tyrimai galėtų būti integruoti į šiuo metu vykstančias arklių infekcinės anemijos stebėsenos programas.

5.6. Išvados

1. Erkinio encefalito viruso (EEV) RNR nustatyta atitinkamai 4,3 proc. (95 proc. PI 3,3–5,5) ir 4,5 proc. (95 proc. PI 2,5–7,4) tirtų ožkų ir avių pieno mėginių. Minimalus infekcijos dažnis ūkių aplinkoje surinktose erkėse buvo 0,3 proc. (95 proc. PI 0,2–0,6). EEV neutralizuojantys antikūnai (IgG) aptikti 37,5 proc. (95 proc. PI 32,2–43,1), o EEV-RNR nustatyta 3,9 proc. (95 proc. PI 2,3–6,8) tirtų arklių kraujo serumo mėginių. Visi mėginiai, kuriuose buvo aptikta EEV-RNR, priklausė Europos EEV potipiui.
2. EEV statistiškai dažniau buvo aptinkamas vidutinio dydžio pieno ūkiuose (6–20 gyvūnų) ($r = 0,54$, $p = 0,01$). Didžiausias viruso kopijų skaičius (4,6 $\log_{10}$ kopijų/ml) nustatytas mažuose ūkiuose (1–5 gyvūnai) ($r = 0,64$, $p < 0,005$). EEV virusas, prieš tai aptiktas piene, 22,4 proc. PI (95 proc. PI 12,5–35,2) mėginių neprarado integralumo ir gebėjo užkrėsti Marc-145 ir Vero ląstelių kultūras. EEV buvo aptiktas 1 proc. (95 proc. PI 0,2–3) visų tirtų pieno produktų, tačiau virusas negebėjo užkrėsti ląstelių kultūrų.

3.1. Pieno mėginių tyrimų strategija yra pranašesnė nei aplinkoje surinktų erkių tyrimai, nes leidžia 6,2 karto dažniau aptikti EEV-RNR geografinėje plotmėje, paplitimo rezultatai sutampa su sergamumo dažniu žmonių tarpe ($r = 0,74$; $p = 0,035$) laiko skalėje ir geografinis pasiskirstymas atitinka žinomas endeminės erkinio encefalito viruso paplitimo ribas.

3.2. Arklių kraujo serologiniai tyrimai gali būti naudojami netiesioginiam erkinio encefalito viruso paplitimo vertinimui, nes geografiškai atitinka endemines EEV paplitimo ribas ir sutampa su žmonių sergamumo duomenimis ($r = 0,76$; $p < 0,05$).

REFERENCES

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

[2] Varlacher J-F, Hägglund S, Juremalm M, et al. Tick-borne encephalitis: -EN- -FR- Encéphalite transmise par les tiques -ES- Encefalitis transmitida por garrapatas. *Rev Sci Tech OIE* 2015; 34: 453–466.

[3] Ilic M, Barbic L, Bogdanic M, 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; 11: 101513.

[4] Caini S, Szomor K, Ferenczi E, et al. Tick-borne encephalitis transmitted by unpasteurised cow milk in western Hungary, September to October 2011. *Eurosurveillance*; 17. Epub ahead of print 22 March 2012. DOI: 10.2807/ese.17.12.20128-en.

[5] Holzmann H, Aberle SW, Stiasny K, et al. Tick-borne Encephalitis from Eating Goat Cheese in a Mountain Region of Austria. *Emerg Infect Dis* 2009; 15: 1671–1673.

[6] Hudopisk N, Korva M, Janet E, et al. Tick-borne Encephalitis Associated with Consumption of Raw Goat Milk, Slovenia, 2012. *Emerg Infect Dis*; 19. Epub ahead of print May 2013. DOI: 10.3201/eid1905.121442.

[7] Markovinović L, Kosanović Ličina ML, Tešić V, et al. An outbreak of tick-borne encephalitis associated with raw goat milk and cheese consumption, Croatia, 2015. *Infection* 2016; 44: 661–665.

[8] Vereta L.A., Skorobrekha V.Z., Nikolaeva S.P., Aleksandrov V.I., Tolstonogova V.I., Zakharycheva T.A., Red'ko A.P., Lev M.I., Savel'eva N.A. The transmission of the tick-borne encephalitis virus via cow's milk. Med. Parazitol. 1991;3:54–56.

[9] Zaludko J., Vrbová O., Hachlincová R., Kohl I., Hubálek Z., Jurincová Z., Kozuch O., Elecková E., Labuda M. Familial epidemics of tick-borne encephalitis in central Povazie. Bratislavske Lekarske Listy. 1994;95:523–526.

[10] Süss J. Tick-borne encephalitis in Europe and beyond – the epidemiological situation as of 2007. *Eurosurveillance*; 13. Epub ahead of print 26 June 2008. DOI: 10.2807/ese.13.26.18916-en.

[11] Kunze U. Tick-borne encephalitis – a notifiable disease. *Ticks and Tick-borne Diseases* 2013; 4: 363–365.

[12] Radzišauskienė D, Žagminas K, Ašoklienė L, 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; 25: 268–274.

[13] European Centre for Disease Prevention and Control. Tick-Borne Encephalitis—Annual Epidemiological Report for 2018; European Centre for Disease Prevention and Control: Stockholm, Sweden, 2019.

[14] European Centre for Disease Prevention and Control. Country Profile: Lithuania. Tick-Borne Encephalitis (TBE); European Centre for Disease Prevention and Control: Stockholm, Sweden, 2012.

[15] Walter M, Vogelgesang JR, Rubel F, et al. Tick-Borne Encephalitis Virus and Its European Distribution in Ticks and Endothermic Mammals. *Microorganisms* 2020; 8: 1065.

[16] *Lindquist, L. Tick-Borne encephalitis. In Handbook of Clinical Neurology; Elsevier Health Sciences: Amsterdam, The Netherlands, 2014; pp. 531–559.*

[17] Erber W, Schmitt H-J. Self-reported tick-borne encephalitis (TBE) vaccination coverage in Europe: Results from a cross-sectional study. *Ticks and Tick-borne Diseases* 2018; 9: 768–777.

[18] Randolph SE, on behalf of the EDEN-TBD sub-project team C. Human activities predominate in determining changing incidence of tick-borne encephalitis in Europe. *Eurosurveillance*; 15. Epub ahead of print 8 July 2010. DOI: 10.2807/ese.15.27.19606-en.
[19] Randolph SE. Tick-borne encephalitis incidence in Central and Eastern Europe: consequences of political transition. *Microbes and Infection* 2008; 10: 209–216.
[20] Daniel M, Kříž B, Danielová V, et al. Correlation between meteorological factors and tick-borne encephalitis incidence in the Czech Republic. *Parasitol Res* 2008; 103: 97–107.
[21] Süss J, Schrader C, Falk U, et al. Tick-borne encephalitis (TBE) in Germany — Epidemiological data, development of risk areas and virus prevalence in field-collected ticks and in ticks removed from humans. *International Journal of Medical Microbiology Supplements* 2004; 293: 69–79.
[22] Stefanoff P, Pfeffer M, Hellenbrand W, 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; 60: 215–226.
[23] Imhoff M, Hagedorn P, Schulze Y, et al. Review: Sentinels of tick-borne encephalitis risk. *Ticks and Tick-borne Diseases* 2015; 6: 592–600.
[24] Achazi K, Růžek D, Donoso-Mantke O, et al. Rodents as Sentinels for the Prevalence of Tick-Borne Encephalitis Virus. *Vector-Borne and Zoonotic Diseases* 2011; 11: 641–647.
[25] Knap N, Korva M, Dolinšek V, et al. Patterns of Tick-Borne Encephalitis Virus Infection in Rodents in Slovenia. *Vector-Borne and Zoonotic Diseases* 2012; 12: 236–242.
[26] Klaus C, Hoffmann B, Beer M, et al. Seroprevalence of tick-borne encephalitis (TBE) in naturally exposed monkeys (Macaca sylvanus) and sheep and prevalence of TBE virus in ticks in a TBE endemic area in Germany. *Ticks and Tick-borne Diseases* 2010; 1: 141–144.
[27] Klaus C, Beer M, Saier R, 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; 3: 27–37.
[28] Duscher GG, Wetscher M, Baumgartner R, et al. Roe deer sera used for TBE surveillance in Austria. *Ticks and Tick-borne Diseases* 2015; 6: 489–493.
[29] Lindhe KE, Meldgaard DS, Jensen PM, et al. Prevalence of tick-borne encephalitis virus antibodies in dogs from Denmark. *Acta Vet Scand* 2009; 51: 56.
[30] Klaus C, Hörügel U, Hoffmann B, et al. Tick-borne encephalitis virus (TBEV) infection in horses: Clinical and laboratory findings and epidemiological investigations. *Veterinary Microbiology* 2013; 163: 368–372.
[31] Rushton JO, Lecollinet S, Hubálek Z, et al. Tick-borne Encephalitis Virus in Horses, Austria, 2011. *Emerg Infect Dis* 2013; 19: 635–637.
[32] Müller K, König M, Thiel HJ. Die tick-borne Enzephalitis (TBE) unter besonderer Berücksichtigung der Infektion beim Pferd [Tick-borne encephalitis (TBE) with special emphasis on infection in horses]. Dtsch Tierarztl Wochenschr. 2006 Apr;113(4):147-51.
[33] Blomqvist G, Näslund K, Svensson L, et al. Mapping geographical areas at risk for tick-borne encephalitis (TBE) by analysing bulk tank milk from Swedish dairy cattle herds for the presence of TBE virus–specific antibodies. *Acta Vet Scand* 2021; 63: 16.
[34] Wallenhammar A, Lindqvist R, Asghar N, et al. Revealing new tick-borne encephalitis virus foci by screening antibodies in sheep milk. *Parasites Vectors* 2020; 13: 185.

[35] Paulsen KM, Stuen S, das Neves CG, et al. Tick-borne encephalitis virus in cows and unpasteurized cow milk from Norway. *Zoonoses Public Health* 2019; 66: 216–222.
[36] Cisak E, Wójcik-Fatla A, Zając V, et al. 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: 283–286.
[37] Füzik T, Formanová P, Růžek D, et al. Structure of tick-borne encephalitis virus and its neutralization by a monoclonal antibody. *Nat Commun* 2018; 9: 436.
[38] Heinz FX, Stiasny K. Flaviviruses and flavivirus vaccines. *Vaccine* 2012; 30: 4301–4306.
[39] Pulkkinen L, Butcher S, Anastasina M. Tick-Borne Encephalitis Virus: A Structural View. *Viruses* 2018; 10: 350.
[40] Gritsun TS, Lashkevich VA, Gould EA. Tick-borne encephalitis. *Antiviral Research* 2003; 57: 129–146.
[41] Calisher CH, Gould EA. Taxonomy of the virus family Flaviviridae. In: *Advances in Virus Research*. Elsevier, pp. 1–19.
[42] Calisher CH. Antigenic classification and taxonomy of flaviviruses (family Flaviviridae) emphasizing a universal system for the taxonomy of viruses causing tick-borne encephalitis. Acta Virol. 1988 Sep;32(5):469-78. PMID: 2904743.
[43] Gritsun TS, Nuttall PA, Gould EA. Tick-borne Flaviviruses. In: *Advances in Virus Research*. Elsevier, pp. 317–371.
[44] Grard G, Moureau G, Charrel RN, et al. Genetic characterization of tick-borne flaviviruses: New insights into evolution, pathogenetic determinants and taxonomy. *Virology* 2007; 361: 80–92.
[45] Ecker M, Allison SL, Meixner T, et al. Sequence analysis and genetic classification of tick-borne encephalitis viruses from Europe and Asia. *Journal of General Virology* 1999; 80: 179–185.
[46] Kovalev SY, Mukhacheva TA. Reconsidering the classification of tick-borne encephalitis virus within the Siberian subtype gives new insights into its evolutionary history. *Infection, Genetics and Evolution* 2017; 55: 159–165.
[47] Dai X, Shang G, Lu S, et al. A new subtype of eastern tick-borne encephalitis virus discovered in Qinghai-Tibet Plateau, China. *Emerging Microbes & Infections* 2018; 7: 1–9.
[48] Deviatkin AA, Karganova GG, Vakulenko YA, et al. TBEV Subtyping in Terms of Genetic Distance. *Viruses* 2020; 12: 1240.
[49] Gould EA, Moss SR, Turner SL. Evolution and dispersal of encephalitic flaviviruses. In: Calisher CH, Griffin DE (eds) *Emergence and Control of Zoonotic Viral Encephalitides*. Vienna: Springer Vienna, pp. 65–84.
[50] Ruzek D, Avšič Županc T, Borde J, et al. Tick-borne encephalitis in Europe and Russia: Review of pathogenesis, clinical features, therapy, and vaccines. *Antiviral Research* 2019; 164: 23–51.
[51] Casati S, Gern L, Piffaretti J-C. Diversity of the population of Tick-borne encephalitis virus infecting Ixodes ricinus ticks in an endemic area of central Switzerland (Canton Bern). *Journal of General Virology* 2006; 87: 2235–2241.
[52] Chitimia-Dobler L, Lemhöfer G, Król N, et al. Repeated isolation of tick-borne encephalitis virus from adult Dermacentor reticulatus ticks in an endemic area in Germany. *Parasites Vectors* 2019; 12: 90.
[53] Dzhivanyan TI, Lashkevich VA, Bannova GG, et al. On the possible association of the DS marker of tick-borne encephalitis virus strains with species of tick vectors. *Archiv f Virusforschung* 1974; 45: 209–214.

[54] Gritsun TS, Frolova TV, Zhankov AI, et al. Characterization of a Siberian Virus Isolated from a Patient with Progressive Chronic Tick-Borne Encephalitis. *J Virol* 2003; 77: 25–36.
[55] Hayasaka D, Ivanov L, Leonova GN, et al. Distribution and characterization of tick-borne encephalitis viruses from Siberia and far-eastern Asia. *Journal of General Virology* 2001; 82: 1319–1328.
[56] Süss J. Epidemiology and ecology of TBE relevant to the production of effective vaccines. *Vaccine* 2003; 21: S19–S35.
[57] Kozuch O, Nosek J. Transmission of tick-borne encephalitis (TBE) virus by Dermacentor marginatus and D. reticulatus ticks. Acta Virol. 1971 Jul;15(4):334. PMID: 4398089.
[58] Kozuch, O.; Nosek, J. Experimental transmission of tick-borne encephalitis (TBE) virus by Haemaphysalis concinna ticks. Acta Virologica 1980, 24, 377.
[59] Nosek, J.; Kozuch, O. Replication of tick-borne encephalitis (TBE) virus in ticks Dermacentor marginatus. Angew. Parasitol. 1985, 26, 97–101.
[60] Belova OA, Litov AG, Kholodilov IS, 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; 8: 895–906.
[61] Mierzejewska EJ, Pawełczyk A, Radkowski M, et al. Pathogens vectored by the tick, Dermacentor reticulatus, in endemic regions and zones of expansion in Poland. *Parasites Vectors* 2015; 8: 490.
[62] Mehlhorn H, Mehlhorn T, Müller M, et al. Tick survey for prevalent pathogens in peri-urban recreation sites in Saarland and Rhineland-Palatinate (Germany). *Parasitol Res* 2016; 115: 1167–1172.
[63] Földvári G, Široký P, Szekeres S, et al. Dermacentor reticulatus: a vector on the rise. *Parasites Vectors* 2016; 9: 314.
[64] Biernat B, Karbowiak G, Werszko J, et al. Prevalence of tick-borne encephalitis virus (TBEV) RNA in Dermacentor reticulatus ticks from natural and urban environment, Poland. *Exp Appl Acarol* 2014; 64: 543–551.
[65] Labuda M, Kozuch O, Zuffová E, et al. Tick-Borne Encephalitis Virus Transmission between Ticks Cofeeding on Specific Immune Natural Rodent Hosts. *Virology* 1997; 235: 138–143.
[66] Randolph SE. Transmission of tick-borne pathogens between co-feeding ticks: Milan Labuda's enduring paradigm. *Ticks and Tick-borne Diseases* 2011; 2: 179–182.
[67] Michelitsch A, Wernike K, Klaus C, et al. Exploring the Reservoir Hosts of Tick-Borne Encephalitis Virus. *Viruses* 2019; 11: 669.
[68] Danielová V, Holubová J, Pejčoch M, et al. Potential significance of transovarial transmission in the circulation of tick-borne encephalitis virus. *FOLIA PARASIT* 2002; 49: 323–325.
[69] Rehacek, J. Transovarial Transmission of Tick-Borne Encephalitis Virus by Ticks. Acta Virol. 1962, 6, 220–226.
[70] Havlíková S, Ličková M, Klempa B. Non-viraemic transmission of tick-borne viruses. *av* 2013; 57: 123–129.
[71] Labuda M, Austyn JM, Zuffova E, et al. Importance of Localized Skin Infection in Tick-Borne Encephalitis Virus Transmission. *Virology* 1996; 219: 357–366.
[72] Ličková M, Fumačová Havlíková S, Sláviková M, et al. Alimentary Infections by Tick-Borne Encephalitis Virus. *Viruses* 2021; 14: 56.
[73] Sauer JR, McSwain JL, Bowman AS, et al. Tick Salivary Gland Physiology. *Annu Rev Entomol* 1995; 40: 245–267.

[74] Karbowiak G, Biernat B. The role of particular tick developmental stages in the circulation of tick-borne pathogens affecting humans in Central Europe. 2. Tick-borne encephalitis virus. *Annals of Parasitology* 2016; 3–9.
[75] Slovák M, Kazimírová M, Siebenstichová M, et al. Survival dynamics of tick-borne encephalitis virus in Ixodes ricinus ticks. *Ticks and Tick-borne Diseases* 2014; 5: 962–969.
[76] Golovljova I, Vene S, Sjölander KB, et al. Characterization of tick-borne encephalitis virus from Estonia: TBE Virus in Estonia. *J Med Virol* 2004; 74: 580–588.
[77] Jaaskelainen A, Tikkakoski T, Uzcategui N, et al. Siberian Subtype Tickborne Encephalitis Virus, Finland. *Emerg Infect Dis* 2006; 12: 1568–1571.
[78] Jaenson TG, Hjertqvist M, Bergström T, et al. Why is tick-borne encephalitis increasing? A review of the key factors causing the increasing incidence of human TBE in Sweden. *Parasites Vectors* 2012; 5: 184.
[79] Estrada-Peña A, Farkas R, Jaenson TGT, et al. Association of environmental traits with the geographic ranges of ticks (Acari: Ixodidae) of medical and veterinary importance in the western Palearctic. A digital data set. *Exp Appl Acarol* 2013; 59: 351–366.
[80] Medlock JM, Hansford KM, Bormane A, et al. Driving forces for changes in geographical distribution of Ixodes ricinus ticks in Europe. *Parasites Vectors* 2013; 6: 1.
[81] Osipova TN, Grigoryeva LA, Samoylova EP, et al. The influence of meteorological factors on the activity of adult taiga ticks (Ixodes persulcatus Sch., Ixodinae) in St. Petersburg and its environs. *Entmol Rev* 2017; 97: 554–563.
[82] Dautel H, Dippel C, Kämmer D, et al. Winter activity of Ixodes ricinus in a Berlin forest. *International Journal of Medical Microbiology* 2008; 298: 50–54.
[83] Buczek A, Bartosik K, Zając Z. Changes in the activity of adult stages of Dermacentor reticulatus (Ixodida: Amblyommidae) induced by weather factors in eastern Poland. *Parasit Vectors* 2014; 7: 245.
[84] Kramer LD, Ebel GD. SHORT REPORT: DURATION OF TICK ATTACHMENT REQUIRED FOR TRANSMISSION OF POWASSAN VIRUS BY DEER TICKS. *The American Journal of Tropical Medicine and Hygiene* 2004; 71: 268–271.
[85] Tonteri E, Kipar A, Voutilainen L, et al. The Three Subtypes of Tick-Borne Encephalitis Virus Induce Encephalitis in a Natural Host, the Bank Vole (Myodes glareolus). *PLoS ONE* 2013; 8: e81214.
[86] Takashima I, Takahashi K, Osada M, et al. Isolation of tick-borne encephalitis virus from wild rodents and a seroepizootiologic survey in Hokkaido, Japan. *The American Journal of Tropical Medicine and Hygiene* 1999; 60: 287–291.
[87] Bakhvalova VN, Dobrotvorsky AK, Panov VV, et al. Natural Tick-Borne Encephalitis Virus Infection among Wild Small Mammals in the Southeastern Part of Western Siberia, Russia. *Vector-Borne and Zoonotic Diseases* 2006; 6: 32–41.
[88] Bakhvalova VN, Potapova OF, Panov VV, et al. Vertical transmission of tick-borne encephalitis virus between generations of adapted reservoir small rodents. *Virus Research* 2009; 140: 172–178.
[89] Burri C, Korva M, Bastic V, et al. Serological Evidence of Tick-Borne Encephalitis Virus Infection in Rodents Captured at Four Sites in Switzerland. *jnl med entom* 2012; 49: 436–439.
[90] Weidmann M, Schmidt P, Hufert FT, et al. Tick-Borne Encephalitis Virus in *Clethrionomys glareolus* in the Czech Republic. *Vector-Borne and Zoonotic Diseases* 2006; 6: 379–381.
[91] Zeman P, Januška J. Epizootiologic background of dissimilar distribution of human cases of Lyme borreliosis and tick-borne encephalitis in a joint endemic area. *Comparative Immunology, Microbiology and Infectious Diseases* 1999; 22: 247–260.

[92] Pintér R, Madai M, Horváth 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; 14: 621–624.

[93] Zöldi V, Papp T, Reiczigel J, et al. Bank voles show high seropositivity rates in a natural TBEV focus in Hungary. *Infectious Diseases* 2015; 47: 178–181.

[94] Roelandt S, Suin V, Riocreux F, et al. Autochthonous Tick-Borne Encephalitis Virus-Seropositive Cattle in Belgium: A Risk-Based Targeted Serological Survey. *Vector-Borne and Zoonotic Diseases* 2014; 14: 640–647.

[95] Šikutová S, Hornok S, Hubálek Z, et al. Serological survey of domestic animals for tick-borne encephalitis and Bhanja viruses in northeastern Hungary. *Veterinary Microbiology* 2009; 135: 267–271.

[96] Rieille N, Klaus C, Hoffmann D, et al. Goats as sentinel hosts for the detection of tick-borne encephalitis risk areas in the Canton of Valais, Switzerland. *BMC Vet Res* 2017; 13: 217.

[97] Rizzoli A, Neteler M, Rosà R, et al. Early detection of tick-borne encephalitis virus spatial distribution and activity in the province of Trento, northern Italy. *Geospat Health* 2007; 1: 169.

[98] Ytrehus B, Vainio K, Dudman SG, et al. Tick-Borne Encephalitis Virus and Louping-Ill Virus May Co-Circulate in Southern Norway. *Vector-Borne and Zoonotic Diseases* 2013; 13: 762–768.

[99] Skarphédinsson S, Jensen PM, Kristiansen K. Survey of Tickborne Infections in Denmark. *Emerg Infect Dis* 2005; 11: 1055–1061.

[100] Balling A, Plessow U, Beer M, et al. Prevalence of antibodies against tick-borne encephalitis virus in wild game from Saxony, Germany. *Ticks and Tick-borne Diseases* 2014; 5: 805–809.

[101] Lindhe KE, Meldgaard DS, Jensen PM, et al. Prevalence of tick-borne encephalitis virus antibodies in dogs from Denmark. *Acta Vet Scand* 2009; 51: 56.

[102] Roelandt S, Heyman P, De Filette M, 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; 11: 1371–1376.

[103] Csángó PA, Blakstad E, Kirtz GC, et al. Tick-borne Encephalitis in Southern Norway. *Emerg Infect Dis* 2004; 10: 533–534.

[104] Dumpis U, Crook D, Oksi J. Tick-Borne Encephalitis. *CLIN INFECT DIS* 1999; 28: 882–890.

[105] Hubálek Z, Rudolf I, Nowotny N. Arboviruses Pathogenic for Domestic and Wild Animals. In: *Advances in Virus Research*. Elsevier, pp. 201–275.

[106] Venturi G, Martelli P, Mazzolini E, et al. Humoral immunity in natural infection by tick-borne encephalitis virus. *J Med Virol* 2009; 81: 665–671.

[107] 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; 225: 173–177.

[108] Lipowski D, Popiel M, Perlejewski K, et al. A Cluster of Fatal Tick-borne Encephalitis Virus Infection in Organ Transplant Setting. *The Journal of Infectious Diseases* 2017; 215: 896–901.

[109] Vaisviliene, D. TBE in Lithuania. In Tickborne Encephalitis and Lyme Borreliosis. 4th International Potsdam Symposium on Tick-Borne Diseases; Süss, J., Kahl, O., Eds.; Pabst Science Publishers: Lengerich, Germany, 1997; pp. 100–113.

[110] Scherer, W.F. Laboratory Safety for Arboviruses and Certain Other Viruses of Vertebrates. Am. J. Trop. Med. Hyg. 1980, 29, 1359–1381.

[111] Avšič-Županc T, Poljak M, Matičič M, et al. Laboratory acquired tick-borne meningoencephalitis: characterisation of virus strains. *Clinical and Diagnostic Virology* 1995; 4: 51–59.
[112] Kräusler, J. Tick-Borne Encephalitis; Facultas: Vienna, Austria, 1981.
[113] Waldenström J, Lundkvist Å, Falk KI, et al. Migrating Birds and Tickborne Encephalitis Virus. *Emerg Infect Dis* 2007; 13: 1215–1218.
[114] Hubálek Z, Rudolf I. Tick-borne viruses in Europe. *Parasitol Res* 2012; 111: 9–36.
[115] Richardson JH, Shore RF, Treweek JR, et al. Are major roads a barrier to small mammals? *Journal of Zoology* 1997; 243: 840–846.
[116] Hasle G. Transport of ixodid ticks and tick-borne pathogens by migratory birds. *Front Cell Infect Microbiol*; 3. Epub ahead of print 2013. DOI: 10.3389/fcimb.2013.00048.
[117] Klaus C, Gethmann J, Hoffmann B, et al. Tick infestation in birds and prevalence of pathogens in ticks collected from different places in Germany. *Parasitol Res* 2016; 115: 2729–2740.
[118] Mansfield KL, Johnson N, Phipps LP, et al. Tick-borne encephalitis virus – a review of an emerging zoonosis. *Journal of General Virology* 2009; 90: 1781–1794.
[119] Gustafson R, Svenungsson B, Forsgren M, et al. Two-year survey of the incidence of lyme borreliosis and tick-borne encephalitis in a high-risk population in Sweden. *Eur J Clin Microbiol Infect Dis* 1992; 11: 894–900.
[120] Lindquist L, Vapalahti O. Tick-borne encephalitis. *The Lancet* 2008; 371: 1861–1871.
[121] Bogovic P. Tick-borne encephalitis: A review of epidemiology, clinical characteristics, and management. *WJCC* 2015; 3: 430.
[122] Adelshin R, Adelshin O, Trushina Y, et al. Tick-Borne Encephalitis Virus Isolates Features from Natural Foci of Pribaikalie (Eastern Siberia, Russia). *J Infect Dis Epidemiol*; 1. Epub ahead of print 31 December 2015. DOI: 10.23937/2474-3658/1510002.
[123] Formanová P, Černý J, Bolfíková BČ, et al. Full genome sequences and molecular characterization of tick-borne encephalitis virus strains isolated from human patients. *Ticks and Tick-borne Diseases* 2015; 6: 38–46.
[124] Jemeršić L, Dežđek D, Brnić D, 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; 5: 7–13.
[125] Kaiser R. Tick-Borne Encephalitis. *Infectious Disease Clinics of North America* 2008; 22: 561–575.
[126] Bogovic P, Lotric-Furlan S, Strle F. What tick-borne encephalitis may look like: Clinical signs and symptoms. *Travel Medicine and Infectious Disease* 2010; 8: 246–250.
[127] Haglund M, Forsgren M, Lindh G, et al. 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; 28: 217–224.
[128] Kaiser R. The clinical and epidemiological profile of tick-borne encephalitis in southern Germany 1994–98. *Brain* 1999; 122: 2067–2078.
[129] Mickiene A, Laiskonis A, Günther G, et al. Tickborne encephalitis in an area of high endemicity in lithuania: disease severity and long-term prognosis. *Clin Infect Dis* 2002; 35: 650–658.
[130] Růžek D, Dobler G, Mantke OD. Tick-borne encephalitis: Pathogenesis and clinical implications. *Travel Medicine and Infectious Disease* 2010; 8: 223–232.
[131] Zindel, W.; Wyler, R. Tick-borne encephalitis in a goat in lower Prätigau. Schweiz. Arch. Tierheilkd. 1983, 125, 383–386.

[132] Böhm B, Schade B, Bauer B, et al. Tick-borne encephalitis in a naturally infected sheep. *BMC Vet Res* 2017; 13: 267.
[133] Bagó Z, Bauder B, Weissenböck H, et al. Tickborne encephalitis in a mouflon (*Ovis ammon musimon*). *Veterinary Record* 2002; 150: 218–220.
[134] Leutloff, R.; Nübling, M.; Neumann-Haefelin, D.; Rieger, M.A. Cows as indicators for TBE endemic regions: Suitability of testing for antibodies in serum and milk. Int. J. Med. Microbiol. 2006, 296, 87–88.
[135] Leschnik MW, Kirtz GC, Thalhammer JG. Tick-borne encephalitis (TBE) in dogs. *International Journal of Medical Microbiology* 2002; 291: 66–69.
[136] Pfeffer M, Dobler G. Tick-borne encephalitis virus in dogs - is this an issue? *Parasites Vectors* 2011; 4: 59.
[137] Zavadska D, Anca I, Andre F, et al. Recommendations for tick-borne encephalitis vaccination from the Central European Vaccination Awareness Group (CEVAG). *Human Vaccines & Immunotherapeutics* 2013; 9: 362–374.
[138] Slunge D, Boman A. Learning to live with ticks? The role of exposure and risk perceptions in protective behaviour against tick-borne diseases. *PLoS ONE* 2018; 13: e0198286.
[139] Slunge D, Jore S, Krogfelt KA, et al. Who is afraid of ticks and tick-borne diseases? Results from a cross-sectional survey in Scandinavia. *BMC Public Health* 2019; 19: 1666.
[140] Jepsen MT, Jokelainen P, Jore S, et al. Protective practices against tick bites in Denmark, Norway and Sweden: a questionnaire-based study. *BMC Public Health* 2019; 19: 1344.
[141] Lesnicar G, Poljak M, Seme K, et al. Pediatric tick-borne encephalitis in 371 cases from an endemic region in Slovenia, 1959 to 2000. *Pediatr Infect Dis J* 2003; 22: 612–618.
[142] Logar M, Bogovič P, Cerar D, et al. Tick-borne encephalitis in Slovenia from 2000 to 2004: Comparison of the course in adult and elderly patients. *Wien Klin Wochenschr* 2006; 118: 702–707.
[143] Bröker M, Gniel D. New foci of tick-borne encephalitis virus in Europe: consequences for travellers from abroad. *Travel Medicine and Infectious Disease* 2003; 1: 181–184.
[144] Rendi-Wagner P. Risk and Prevention of Tick-borne Encephalitis in Travelers. *Journal of Travel Medicine* 2006; 11: 307–312.
[145] European Centre for Disease Prevention and Control (ECDC). EU case definitions: European Commission; 2018. Available at: https://ecdc.europa.eu/en/surveillance-and-disease-data/eu-case- definitions.
[146] Tick-borne encephalitis. Annual Epidemiological Report for 2019. Available at: https://www.ecdc.europa.eu/sites/default/files/documents/AER-TBE-2019.pdf.
[147] European Centre for Disease Prevention and Control (ECDC). Tick-borne encephalitis (TBE): factsheet for health professionals Stockholm: ECDC; 2010 Available at: https://ecdc.europa.eu/en/tick-borne-encephalitis/facts/factsheet.
[148] Kreusch TM, Holding M, Hewson R, et al. A probable case of tick-borne encephalitis (TBE) acquired in England, July 2019. *Eurosurveillance*; 24. Epub ahead of print 21 November 2019. DOI: 10.2807/1560-7917.ES.2019.24.47.1900679.
[149] Holding M, Dowall S, Hewson R. Detection of tick-borne encephalitis virus in the UK. *The Lancet* 2020; 395: 411.
[150] Holding M, Dowall SD, Medlock JM, et al. Detection of new endemic focus of tick-borne encephalitis virus (TBEV), Hampshire/Dorset border, England, September 2019. *Eurosurveillance*; 24. Epub ahead of print 21 November 2019. DOI: 10.2807/1560-7917.ES.2019.24.47.1900658.
[151] ECDC Surveillance Atlas. Available at: http://atlas.ecdc.europa.eu/public/index.aspx.

[152] Kríz B, Benes C, Daniel M. Alimentary transmission of tick-borne encephalitis in the Czech Republic (1997-2008). Epidemiol Mikrobiol Imunol. 2009 Apr;58(2):98-103. PMID: 19526924.
[153] Amicizia D, Domnich A, Panatto D, et al. Epidemiology of tick-borne encephalitis (TBE) in Europe and its prevention by available vaccines. *Human Vaccines & Immunotherapeutics* 2013; 9: 1163–1171.
[154] Brockmann S, Oehme R, Buckenmaier T, et al. A cluster of two human cases of tick-borne encephalitis (TBE) transmitted by unpasteurised goat milk and cheese in Germany, May 2016. *Eurosurveillance*; 23. Epub ahead of print 12 April 2018. DOI: 10.2807/1560-7917.ES.2018.23.15.17-00336.
[155] Blaškovic, D. Epidémia Encefalitídy v rožnˇavskom Prírodnom Ohnisku Nákaz Sborník Príspevkov o Epidémii Encefalitídy Prenesenej Mliekom a o Komplexnom Výskume Prírodného Ohniska Nákaz, 1st ed.; Vydavatel'stvo Slovenskej Akadémie Vied: Bratislava, Slovakia, 1954.
[156] Labuda M, Elečková E, Ličková M, et al. Tick-borne encephalitis virus foci in Slovakia. *International Journal of Medical Microbiology* 2002; 291: 43–47.
[157] Kerlik J, Avdičová M, Štefkovičová M, et al. Slovakia reports highest occurrence of alimentary tick-borne encephalitis in Europe: Analysis of tick-borne encephalitis outbreaks in Slovakia during 2007–2016. *Travel Medicine and Infectious Disease* 2018; 26: 37–42.
[158] Balogh Z, Ferenczi E, Szeles K, et al. Tick-borne encephalitis outbreak in Hungary due to consumption of raw goat milk. *Journal of Virological Methods* 2010; 163: 481–485.
[159] Zöldi, V.; Ferenczi, E.; Egyed, L. Milk Transmitted Tick-Borne Encephalitis Epidemics in Hungary. Magy. Állatorvosok Lapja 2013, 135, 48–56.
[160] Zöldi V, Juhász A, Nagy C, et al. Tick-Borne Encephalitis and Lyme Disease in Hungary: The Epidemiological Situation Between 1998 and 2008. *Vector-Borne and Zoonotic Diseases* 2013; 13: 256–265.
[161] Pazdiora P, Štruncová V, Švecová M. Tick-borne encephalitis in children and adolescents in the Czech Republic between 1960 and 2007. *World J Pediatr* 2012; 8: 363–366.
[162] Matuszczyk, I.; Tarnowska, H.; Zabicka, J.; Gut, W. The Outbreak of an Epidemic of Tick-Borne Encephalitis in Kielec Province Induced by Milk Ingestion. Prz. Epidemiol. 1997, 51, 381–388.
[163] Król ME, Borawski B, Nowicka-Ciełuszecka A, et al. Outbreak of alimentary tick-borne encephalitis in Podlaskie voivodeship, Poland. *Przegl Epidemiol* 2019; 239–248.
[164] Kerbo N, Donchenko I, Kutsar K, et al. Tickborne encephalitis epidemiology in Estonia, 1950-2004. *Weekly releases (1997–2007)*; 10. Epub ahead of print 30 June 2005. DOI: 10.2807/esw.10.26.02740-en.
[165] Donchenko I, Kutsar K, Vasilenko V, et al. Tickborne encephalitis outbreak in Estonia linked to raw goat milk, May-June 2005. *Weekly releases (1997–2007)*; 10. Epub ahead of print 23 June 2005. DOI: 10.2807/esw.10.25.02730-en.
[166] Chitimia-Dobler L, Lindau A, Oehme R, et al. Tick-Borne Encephalitis Vaccination Protects from Alimentary TBE Infection: Results from an Alimentary Outbreak. *Microorganisms* 2021; 9: 889.
[167] Molnár, G.B.; Persecă, T.; Feder, A.; Păcuraru, D.; Marialaki, E.; Cojan, A. Epidemiological Assessment of Morbidity and Natural Foci of TBE-CEE Virus Infection in Transylvania. Rev. Med. Chir. Soc. Med. Nat. Din Iasi 2008, 112, 471–477.

[168] *Higgins JPT, Thomas J, Chandler J, Cumpston M, Li T, Page MJ, Welch VA (editors). Cochrane Handbook for Systematic Reviews of Interventions. 2nd Edition. Chichester (UK): John Wiley & Sons, 2019.*
[169] Jayarao BM, Donaldson SC, Straley BA, et al. A Survey of Foodborne Pathogens in Bulk Tank Milk and Raw Milk Consumption Among Farm Families in Pennsylvania. *Journal of Dairy Science* 2006; 89: 2451–2458.
[170] Giacometti F, Serraino A, Finazzi G, et al. Field handling conditions of raw milk sold in vending machines: experimental evaluation of the behaviour of *Listeria monocytogenes, Escherichia coli* O157:H7, *Salmonella Typhimurium* and *Campylobacter jejuni*. *Italian Journal of Animal Science* 2012; 11: e24.
[171] Singh GM, Micha R, Khatibzadeh S, et al. Global, Regional, and National Consumption of Sugar-Sweetened Beverages, Fruit Juices, and Milk: A Systematic Assessment of Beverage Intake in 187 Countries. *PLoS ONE* 2015; 10: e0124845.
[172] *World Food and Agriculture - Statistical Yearbook 2020*. FAO. Epub ahead of print 2020. DOI: 10.4060/cb1329en.
[173] FAOSTAT, Food Supply - Livestock and Fish Primary Equivalent. Available from: https://www.fao.org/faostat/en/#data/CL.
[174] Tonteri E, Jääskeläinen AE, Tikkakoski T, et al. Tick-borne Encephalitis Virus in Wild Rodents in Winter, Finland, 2008–2009. *Emerg Infect Dis* 2011; 17: 72–75.
[175] Zöldi V, Papp T, Rigó K, et al. A 4-Year Study of a Natural Tick-Borne Encephalitis Virus Focus in Hungary, 2010–2013. *EcoHealth* 2015; 12: 174–182.
[176] Gerth H-J, Grimshandl D, Stage B, et al. Roe deer as sentinels for endemicity of tick-borne encephalitis virus. *Epidemiol Infect* 1995; 115: 355–365.
[177] Carpi G, Cagnacci F, Neteler M, et al. Tick infestation on roe deer in relation to geographic and remotely sensed climatic variables in a tick-borne encephalitis endemic area. *Epidemiol Infect* 2008; 136: 1416–1424.
[178] Knap N, Avšič-Županc T. Correlation of TBE Incidence with Red Deer and Roe Deer Abundance in Slovenia. *PLoS ONE* 2013; 8: e66380.
[179] Vor T, Kiffner C, Hagedorn P, et al. Tick burden on European roe deer (Capreolus capreolus). *Exp Appl Acarol* 2010; 51: 405–417.
[180] Kriz B, Daniel M, Benes C, et al. 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; 14: 801–807.
[181] Cagnacci F, Bolzoni L, Rosà R, 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; 42: 365–372.
[182] Benda R. Experimental transmission of the Czechoslovak tick-borne encephalitis to goats from infected sucking females of the tick Ixodes ricinus. Cesk. Epidemiol. Mikrobiol. Imunol. 1958, 7, 1–5.
[183] 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; 27: 136–145.
[184] Albornoz A, Carletti T, Corazza G, et al. The Stress Granule Component TIA-1 Binds Tick-Borne Encephalitis Virus RNA and Is Recruited to Perinuclear Sites of Viral Replication To Inhibit Viral Translation. *J Virol* 2014; 88: 6611–6622.
[185] Puchhammer-Stöckl E, Kunz C, Mandl CW, et al. 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; 4: 321–326.
[186] Lundkvist K, Vene S, Golovljova I, et al. Characterization of tick-borne encephalitis virus from latvia: Evidence for co-circulation of three distinct subtypes. *J Med Virol* 2001; 65: 730–735.

[187] Deviatkin AA, Kholodilov IS, Belova OA, et al. Baltic Group Tick-Borne Encephalitis Virus Phylogeography: Systemic Inconsistency Pattern between Genetic and Geographic Distances. *Microorganisms* 2020; 8: 1589.
[188] Grešíková M, Sekeyová M, Stúpalová S, et al. Sheep Milk-Borne Epidemic of Tick-Borne Encephalitis in Slovakia. *Intervirology* 1975; 5: 57–61.
[189] Dorko E, Hockicko J, Rimárová K, et al. Milk outbreaks of tick-borne encephalitis in Slovakia, 2012-2016. *Cent Eur J Public Health* 2018; 26: S47–S50.
[190] van den Brom R, de Jong A, van Engelen E, et al. Zoonotic risks of pathogens from sheep and their milk borne transmission. *Small Ruminant Research* 2020; 189: 106123.
[191] Sidorenko M, Radzijevskaja J, Mickevičius S, et al. Prevalence of tick-borne encephalitis virus in questing Dermacentor reticulatus and Ixodes ricinus ticks in Lithuania. *Ticks and Tick-borne Diseases* 2021; 12: 101594.
[192] Sergamumo užkrečiamosiomis ligomis Lietuvoje 2018 M. apžvalga. Užkrečiamųjų ligų ir AIDS centras.
[193] Sergamumo užkrečiamosiomis ligomis Lietuvoje 2019 M. apžvalga. Užkrečiamųjų ligų ir AIDS centras.
[194] Rieille N, Bressanelli S, Freire CCM, 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: 443.
[195] Lommano E, Burri C, Maeder G, 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; 49: 156–164.
[196] Offerdahl DK, Clancy NG, Bloom ME. Stability of a Tick-Borne Flavivirus in Milk. *Front Bioeng Biotechnol*; 4. Epub ahead of print 11 May 2016. DOI: 10.3389/fbioe.2016.00040.
[197] Balogh Z, Egyed L, Ferenczi E, et al. Experimental Infection of Goats with Tick-Borne Encephalitis Virus and the Possibilities to Prevent Virus Transmission by Raw Goat Milk. *Intervirology* 2012; 55: 194–200.
[198] Drozdov, S. G. Nature of Diphasic Milk Fever; Data on the Focus in the European Part of the USSR. I. Isolation of the Causative Virus and Its Characteristics. Vopr. Virusol. 1959, 4, 204–208.
[199] Gresikova, M. Recovery of the Tick-Borne Encephalitis Virus from the Blood and Milk of Subcutaneously Infected Sheep. Acta Virol. 1958, 2, 113–119.
[200] Randolph SE, Asokliene L, Avsic-Zupanc T, et al. Variable spikes in tick-borne encephalitis incidence in 2006 independent of variable tick abundance but related to weather. *Parasites Vectors* 2008; 1: 44.
[201] Bormane A, Lucenko I, Duks A, et al. Vectors of tick-borne diseases and epidemiological situation in latvia in 1993–2002. *International Journal of Medical Microbiology Supplements* 2004; 293: 36–47.
[202] 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; 3: 240–246.
[203] Van Tongeren HA. Encephalitis in Austria. IV. Excretion of virus by milk of the experimentally infected goat. *Arch Gesamte Virusforsch 1955;6(2-3):158-62*.
[204] Oehme R, Hartelt K, Backe H, et al. Foci of tick-borne diseases in Southwest Germany. *International Journal of Medical Microbiology* 2002; 291: 22–29.
[205] Gäumann R, Mühlemann K, Strasser M, et al. High-Throughput Procedure for Tick Surveys of Tick-Borne Encephalitis Virus and Its Application in a National Surveillance Study in Switzerland. *Appl Environ Microbiol* 2010; 76: 4241–4249.

[206] Piesman J, Eisen L. Prevention of Tick-Borne Diseases. *Annu Rev Entomol* 2008; 53: 323–343.
[207] Updated guidelines for evaluating public health surveillance systems; recommendations from the Guidelines Working Group.
[208] Gilbert M, Nicolas G, Cinardi G, et al. Global distribution data for cattle, buffaloes, horses, sheep, goats, pigs, chickens and ducks in 2010. *Sci Data* 2018; 5: 180227.
[209] Wurm R, Dobler G, Peters M, et al. Serological Investigations of Red Foxes (Vulpes vulpes L.) for Determination of the Spread of Tick-borne Encephalitis in Northrhine-Westphalia. *J Vet Med Series B* 2000; 47: 503–509.
[210] Klaus C, Ziegler U, Kalthoff D, et al. Tick-borne encephalitis virus (TBEV) – findings on cross reactivity and longevity of TBEV antibodies in animal sera. *BMC Vet Res* 2014; 10: 78.
[211] Klaus C, Beer M, Saier R, Schubert H, Bischoff S, Süss J. Evaluation of serological tests for detecting tick-borne encephalitis virus (TBEV) antibodies in animals. Berl Munch Tierarztl Wochenschr. 2011 Nov-Dec;124(11-12):443-9. PMID: 22191165.

LIST OF PUBLICATIONS

This PhD thesis is based on the following publications:

1. **Pautienius Arnoldas**, Austeja Armonaite, Evelina Simkute, Ruta Zagrabskaite, Jurate Buitkuviene, Russell Alpizar-Jara, Juozas Grigas, Indre Zakiene, Dainius Zienius, Algirdas Salomskas, and Arunas Stankevicius. 2021. "Cross-Sectional Study on the Prevalence and Factors Influencing Occurrence of Tick-Borne Encephalitis in Horses in Lithuania" *Pathogens* 10, no. 2: 140. [IF: 3.492].
2. **Pautienius Arnoldas**, Gytis Dudas, Evelina Simkute, Juozas Grigas, Indre Zakiene, Algimantas Paulauskas, Austeja Armonaite, Dainius Zienius, Evaldas Slyzius, and Arunas Stankevicius. 2021. "Bulk Milk Tank Samples Are Suitable to Assess Circulation of Tick-Borne Encephalitis Virus in High Endemic Areas" *Viruses* 13, no. 9: 1772. [IF: 5.048].

This PhD thesis is based on the following abstracts in international or national scientific events:

1. **Pautienius Arnoldas**, Simkute Evelina, Grigas Juozas, Jasineviciute Evelina, Alpizal-Jara Russel, Stankevicius Arunas. Development of Animal-based Approach to Access the Spatial Prevalence of Tick-borne encephalitis. General Scientific Meeting of IMAAC, Lisbon, Portugal, 8 March 2022
2. **Pautienius Arnoldas**, Simkute Evelina, Grigas Juozas, Stankevicius Arunas. Towards the Development of New Surveillance Approach of Tick-Borne Encephalitis Virus // The First Conference of the World Society of Virology (WSC) "Tackling Global Viral Epidemics": 16–18 June 2021
3. **Pautienius Arnoldas**, Simkute Evelina, Grigas Juozas, Stankevicius Arunas. Potential new approach to surveillance strategy of Tick-borne encephalitis virus. 3rd Training School on Optimal Control Theory, Mathematical Modelling and Mosquito Control Strategies, Fuerteventura, Canary Islands/Spain. 2–4 March 2020
4. **Pautienius Arnoldas**, Šimkute Evelina, Grigas Juozas, Stankevičius Arūnas. Erkinio encefalito viruso paplitimas Lietuvoje ir naujo stebėsenos metodo plėtojimas. 9-oji jaunųjų mokslininkų konferencija „Jaunieji mokslininkai – žemės ūkio pažangai" : pranešimų tezės : [2020 m. lapkričio 12 d.] / Lietuvos mokslų akademijos Žemės ūkio ir miškų mokslų skyrius;. Vilnius : Lietuvos mokslų akademija, 2020

COPIES OF PUBLICATIONS

Brief Report

Bulk Milk Tank Samples Are Suitable to Assess Circulation of Tick-Borne Encephalitis Virus in High Endemic Areas

Arnoldas Pautienius [1,2,*], Gytis Dudas [3], Evelina Simkute [2], Juozas Grigas [1,2], Indre Zakiene [2], Algimantas Paulauskas [4], Austeja Armonaite [2], Dainius Zienius [5], Evaldas Slyzius [6] and Arunas Stankevicius [2]

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Abstract: A reliable surveillance strategy of tick-borne encephalitis virus (TBEV) is necessary to ensure adequate disease control measures. However, current approaches assessing geographical TBEV circulation are ineffective or have significant limitations. In this study we investigated a total of 1363 goat and 312 sheep bulk tank milk samples for the presence of TBEV. Samples were collected from systematically selected farms in Lithuania every 4–5 days from April to November in 2018 and 2019. To validate results, we additionally tested 2685 questing ticks collected in the vicinity of milk collection sites. We found 4.25% (95% CI 3.25–5.47) and 4.48% (95% CI 2.47–7.41) goat and sheep milk samples to be positive for TBEV, respectively. Furthermore, geographical distribution of TBEV in milk samples coincided with the known TBE endemic zone and was correlated with incidence of TBE in humans in 2019. When sampling time coincides, TBEV detection in milk samples is as good a method as via flagged ticks, however bulk milk samples can be easier to obtain more frequently and regularly than tick samples. The minimal infectious rate (MIR) in ticks was 0.34% (CI 95% 0.15–0.64). Therefore, our results confirm that testing milk serves as a valuable tool to investigate the spatial distribution of TBEV at higher resolution and lower cost.

Keywords: TBEV; TBEV in milk; alimentary TBE; TBEV prevalence; tick-borne encephalitis; flavivirus

1. Introduction

Tick-borne encephalitis virus (TBEV) is a zoonotic flavivirus that is considered to be the most important causative agent of tick-borne infections in Europe [1]. TBEV is maintained in nature by *Ixodes* ticks that serve as vectors and constitute the central reservoir for the virus [2]. Although TBEV is usually transmitted to humans through the bites of infected ticks, alimentary infection via unpasteurized milk or milk products has been recognized as an additional route of transmission. In most alimentary cases, TBEV is transmitted after consumption of goat milk, but infection through cow or sheep milk has been reported as well [3–7].

Targeted disease control measures need to be taken based on known virus prevalence. However, human surveillance alone is not enough to effectively monitor the circulation of

TBEV, since national health authorities of European countries use non-uniform TBE case definitions and TBE risk assessment strategies [8]. Moreover, such estimations may be affected by uneven vaccination coverage [9]. Socioeconomic, political, environmental, or meteorological factors may also play a role in determining exposure risk [10–13].

Detection of TBEV in ticks by the flagging and dragging method is known as the common method to assess TBEV circulation [14]. However, the spatiotemporal mismatch between TBEV prevalence in ticks and clinical case notifications in humans has been reported as the main caveat associated with this method [15].

Milk sample testing has been suggested as a promising alternative to determine TBEV prevalence [2]. Two antibody screening investigations of milk in Sweden confirmed that it showed great applicability in mapping high-risk areas [16,17]. However, to the best of our knowledge, only two studies explicitly aiming to detect TBEV itself have been performed, both of which looking at just over a hundred samples [18,19].

Given the high prevalence of TBEV and the common occurrence of foodborne infections, we hypothesize that milk sample testing may help to assess the prevalence of the virus in the environment and may serve as a complement to ongoing monitoring efforts. Therefore, the overall rationale of our study was to evaluate the prevalence of the virus in the milk of small ruminants in Lithuania, where TBEV is endemic throughout the country, and to investigate whether milk sample testing can be adopted as a TBEV surveillance strategy.

2. Materials and Methods

2.1. Sample Collection

Goat and sheep milk samples were collected because of their known or suspected association with food-borne outbreaks, as well as favorable density and homogenous geographical distribution in Lithuania.

The sampling frame was based on data from the National Livestock Register database of Lithuania. Farms were selected from each municipality according to a stratified random sample collection strategy and were included in the study if the following criteria were met: the owners agreed to participate in the study; animals were not vaccinated against any flaviviruses and were not acaricide-treated; farms did not apply consistent environmental tick control measures, and they were not in an urban area.

To avoid missing viremic episodes, all milk samples were collected once every 4–5 days from bulk milk tanks throughout the lactation period between April and November in 2018 and 2019. Each sample of 10–15 mL in volume was stored frozen at −20 °C until further use. We analyzed time intervals between positive samples and measured their viral load, thus removing all consecutive positive samples attributable to a single infection.

Simultaneously, ticks were collected by the flagging and dragging method on farms or in their vicinity, in both 2018 and 2019, at a single point in time between April and November. Ticks were pooled according to development stage, sex, species, and sampling site. Up to 10 *Ixodes ricinus* adults, 20 nymphs, 50 larvae, or 5 *Dermacentor reticulatus* were grouped per pool. After collection, ticks were maintained alive until reaching the laboratory.

Data on human TBE incidence were obtained from the Centre for Communicable Diseases and AIDS of Lithuania.

2.2. TBEV Detection and Viral Load Quantification

The fat fraction of the milk was removed as described elsewhere [19]. Ticks were dissected and homogenized in liquid nitrogen and ground to a fine powder using a mortar and pestle.

Viral RNA from skimmed milk or centrifuged tick supernatant was extracted using GeneJET RNA Purification Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Samples were tested by RT-PCR for the presence of TBEV-specific RNA using primers described previously [20]. PCR-positive samples were

confirmed by partial genome sequencing targeting NCR region of TBEV using primers described before [21]. Reaction mix SuperScript™ III One-Step RT-PCR System with Platinum™ *Taq* DNA Polymerase (Thermo Scientific, Waltham, MA, USA) was used for real time PCR and DreamTaq Green PCR Master Mix (2×) (Thermo Scientific, Waltham, MA, USA) for conventional PCR.

Viral load was determined using a quantification assay, whereby sample concentration was assessed using a calibrated standard curve derived from measurements of serial dilutions of TBE virus samples with known concentrations. All reactions were carried out in triplicate and values averaged.

Quality assessment of RNA extraction of tick samples was performed in the same way as a previous study (20).

2.3. TBEV Isolation

Vero (ATCC® CCL-81™, Manassas, VA, USA) cells were inoculated with 300 μL aliquots of microfiltrated TBEV-RNA positive suspensions. After 1 h incubation, suspensions were discarded, cells were washed with PBS and cultured at 37 °C in 5% CO_2 in Minimum Essential Medium with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 100 U mL^{-1} penicillin and 100 μg L^{-1} streptomycin. Cytopathic effects were examined over five serial passages and performed in triplicate for each round. The success of isolation was assessed by RT-PCR followed after RNA extraction.

3. Results

3.1. TBEV Prevalence in Milk

A total of 1363 goat and 312 sheep unpasteurized bulk milk samples taken from 17 and 4 farms, respectively, were examined for the presence of TBEV RNA.

Overall, 62 (4.54%) and 14 (4.48%) goat and sheep bulk milk samples, respectively, were confirmed positive for TBEV. However, two cases of three consecutive positive samples per farm were also identified. Based on gradual decrease in viral load we determined these cases to be two individual infections. All remaining samples appeared to be positive with a minimal interval of 10–11 days to a maximum range of 3 months. Therefore, the overall number of TBEV positive samples was adjusted to 58/1363 (4.25%, 95% CI 3.25–5.47) for goat and 14/312 (4.48%, 95% CI 2.47–7.41) for sheep samples. At least one positive sample was detected in 70.58% and 64.70% of tested goat farms in 2018 and 2019, respectively. At least one positive sample was detected in 75% of sheep farms during both years of the study.

Though sheep milk sample sizes were small, they demonstrated the same prevalence pattern seen in goat milk samples. Thus, statistical analysis was performed using only the goat samples, but also recalculated using all data. Due to low statistical power, no differences between species were identified.

A summary of sample collection sites and TBEV spatial distribution is presented in Figure 1. To ensure privacy, geographic coordinates of each farm were randomly shifted up to 0.1° from their actual locations. Analysis of the geographic distribution shows that positive samples are fairly evenly distributed throughout the territory of Lithuania and there is no statistical association indicating that any particular area is at higher risk of TBEV.

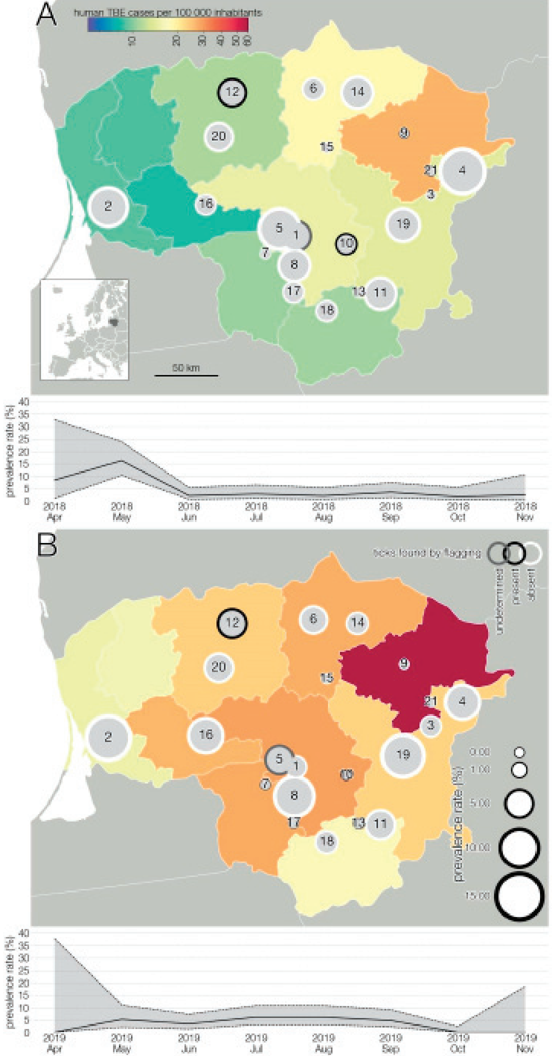


Figure 1. The geographic location of the study area and spatio-longitudinal distribution of TBEV positive cases in small ruminant farms. Results of 2018 (**A**), results of 2019 (**B**). The size of circles indicates TBEV prevalence rate in a given farm. Number 1–17—goat farms; 18–21—sheep farms. Colored administrative units indicate human TBE cases at NUTS3 level.

TBEV prevalence, as detected, fluctuated in time (Figure 1), and varied during the two lactation periods (Figure 2). However, limited data precluded a meaningful interpretation of the effect of time on the occurrence of positive samples. Temporal analysis showed a

significant association between monthly virus prevalence in animals and TBE incidence rates in humans in 2019 (Figure 3). The same correlation in 2018 (r = 0.65) was not significant but showed a trend toward significance ($p = 0.083$).

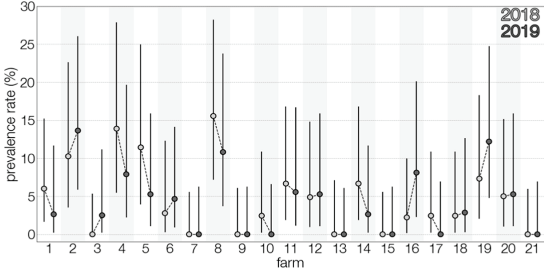


Figure 2. Distribution of TBEV prevalence rates amongst tested farms between the two years of study.

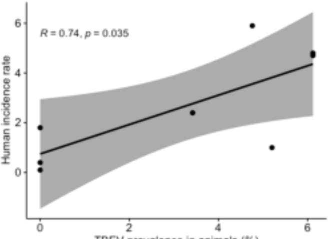


Figure 3. Correlation between TBEV prevalence in animals and human incidence rate (2019 data were used for analysis).

A non-linear correlation was observed between TBEV prevalence and farm sizes (6–20 > 1–5 > more than 21 animals) ($r = -0.54$, $p = 0.01$). Moreover, a linear correlation was observed between virus load and milk amount produced in the farm ($r = -0.64$, $p < 0.005$) (Supplementary Material S1). Results of viral loads are presented in Figure 4.

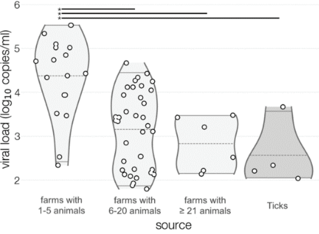
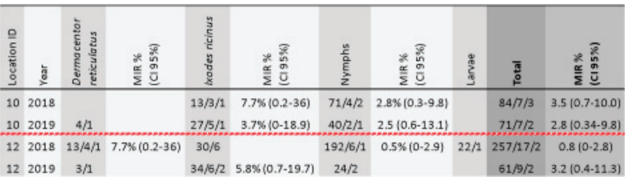


Figure 4. Viral load expressed as $\log_{10}$ viral RNA copies/mL. Asterisks and horizontal lines at the top of violin plots indicate statistically significant difference ($p < 0.05$) in viral load based on Tukey's honest significance test.

3.2. TBEV Prevalence in Ticks

A total of 2685 questing ticks were collected corresponding to 886 adults, 1329 nymphs, and 88 larvae of *Ixodes ricinus*, and 382 *Dermacentor reticulatus* adults. No ticks were found in 3 and 4 sampling locations in 2018 and 2019, respectively.

Of the tested 283 tick pools, we found nine pools positive for TBEV-RNA, corresponding to only two collection sites in which positive samples were detected in both years of the study. Positive tick cases are summarized in Figure 5. The overall minimum infectious rate (MIR) was 0.34% (CI 95% 0.15–0.64).



Location ID	Year	*Dermacentor reticulatus*	MIR % (CI 95%)	*Ixodes ricinus*	MIR % (CI 95%)	Nymphs	MIR % (CI 95%)	Larvae	**Total**	**MIR % (CI 95%)**
10	2018			13/3/1	7.7% (0.2-36)	71/4/2	2.8% (0.3-9.8)		84/7/3	3.5 (0.7-10.0)
10	2019	4/1		27/5/1	3.7% (0-18.9)	40/2/1	2.5 (0.6-13.1)		71/7/2	2.8 (0.34-9.8)
12	2018	13/4/1	7.7% (0.2-36)	30/6		192/6/1	0.5% (0-2.9)	22/1	257/17/2	0.8 (0-2.8)
12	2019	3/1		34/6/2	5.8% (0.7-19.7)	24/2			61/9/2	3.2 (0.4-11.3)

Figure 5. Summary of TBEV positive sample characteristics in ticks. Location ID refers to the map in Figure 1. The sequence of three numbers is explained as follows: total tick sample size/number of pools/number of positive pools.

Amplification of 16S rRNA was successful in all randomly selected pools, confirming there were no false negative results due to inhibition of the PCR assay by tick-originated products.

Specificity of both tick and milk PCR positive sample product was confirmed by partial genome sequencing based on the NCR fragment of the TBEV genome (Figure S1). Sequences of TBEV strains, including Neudörfl, U27495; Sofjin, AB062064, and Vasilchenko, AF069066 were used for phylogenetic comparisons. Analysis showed that all detected TBEV strains belong to the European subtype. Sequences have been submitted to GenBank under accessions MZ664211-MZ664256.

3.3. Comparison of TBEV Detection Methods

Marginal homogeneity between two TBEV surveillance approaches was assessed by McNemar's test. Cases when ticks were not collected on site due to their scarcity were excluded from the contingency table. To compare the capacity of flagging and dragging and bulk milk methods to detect TBEV, milk samples nearest to tick samples in time were analyzed. The data provided no evidence to reject the null hypothesis, thus implying both methods are capable of detecting TBEV when sampling times coincide.

However, milk sampling showed greater effectiveness in terms of time and personnel resources. By our generalized calculations, collection of one milk sample did not take more than 5 min as they were voluntarily collected by the farmers themselves. Their periodic collection and delivery to the laboratory time was also short, due to the small size of the country and optimized travel routes. A brief comparison of the two testing approaches is presented in Figure 6. Nevertheless, these results are highly country-specific and, therefore, should be judged accordingly.

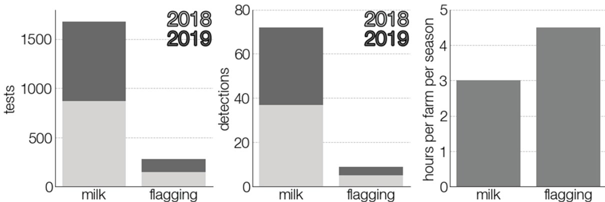


Figure 6. Comparison of two TBEV surveillance strategies. The time spent on sample collection is calculated assuming that it takes two and a half hours for one person to collect ticks and another two hours for tick characterization and pool formation.

3.4. Virus Isolation

To confirm the presence of infective virus, all positive samples were inoculated on Vero cells that were examined for occurrence of cytopathic effect (CPE) characterized by lysis of the cell monolayer. From cultures with no visible CPE, additional sub-passages were carried out. Cells were harvested after 4–7 days. Overall, 6/9 (66%, 95% CI 29.9–92.5) tick homogenates and 13/58 (22.4%, 95% CI 12.5–35.2) goat milk suspensions were successfully isolated and caused CPE beginning 4–6 days post-infection (p.i.). Only one TBEV isolate from sheep milk was successfully isolated. Virus load varied significantly depending on passage number and showed 1–3 $\log_{10}$ increase at final passage (data not shown).

4. Discussion

In this study, a novel and reliable approach for monitoring the prevalence of TBEV is presented. Herein, we conceptualized an epidemiological monitoring strategy where bulk milk tank samples were collected once every 4–5 days to include the time period when the virus is no longer shed through milk, based on previous studies showing that TBEV is detectable in milk for 3-8 days p.i. [22]. Evidence from other studies showed that TBEV may be detectable for a slightly longer period [23,24]. As our results showed, the number of positive samples could be overestimated only to a very limited extent because of the possible misclassification of multiple samples of single infection as separate cases.

TBEV surveillance by tick flagging was carried out in parallel to the longitudinal study of milk samples. TBEV surveillance by tick flagging typically involves collecting ticks from a site once per season, whereas the proposed TBEV surveillance through longitudinal milk sample testing involved collecting approximately 80 milk samples at regular intervals during the season (April to November). The surveillance strategy based on milk sample testing proved to be more reliable as it allowed the detection of TBEV circulation over a wider geographical area, resulting in approximately two thirds of tested sites being positive, contrary to the tick flagging method where only two sites were positive for TBEV (11.4% of tested locations). Furthermore, the wide geographical distribution of TBEV in milk samples coincided with the known area of TBE endemicity, and monthly prevalence of TBEV in milk samples statistically correlated with monthly human incidence rate in one of the two investigated years (2019). The statistical association between aforementioned values was not observed in 2018 due to the remarkably high TBEV prevalence in milk in May, which in turn can possibly be explained by exceptionally favorable environmental conditions affecting the development of ticks.

In agreement with our results, a very similar TBEV prevalence pattern in ticks and almost identical overall MIR was observed in a nationwide study conducted in Lithuania in 2017–2019 where almost 9000 ticks were tested [25]. The latter study also showed a patchy distribution of TBEV with many administrative units apparently free of the virus. These results add weight to previous findings of spatio-temporal patchiness of TBEV in ticks and discordance between numbers of clinical TBE cases in humans and prevalence in ticks [15,26–29].

Unfortunately, straightforward statistical comparisons of the two testing approaches were not possible due to different study designs. Data from our analysis, however, show that both methods allow us to determine the presence of the virus in the environment with reasonable accuracy when they are carried out in close proximity. Thus, it can be said that the flagging and dragging method is a sensitive method to calculate TBEV prevalence in an area but only if considerably high numbers of ticks are tested, which in turn requires unreasonably high financial and personnel resources. This is an important aspect to highlight in the context of numerous studies that have failed to detect the virus in known TBE foci, even when approximately ten or twenty thousand ticks were tested [25,26].

The flagging and dragging method has more drawbacks. In addition to those discussed by other authors, such as low TBEV prevalence rates, great spatiotemporal variability, and time-consuming and labor-intensive sample collection [30–33], we also want to highlight the issue of the risk of being bitten, particularly in areas where Lyme borreliosis is prevalent and against which a vaccine does not yet exist.

TBEV ecology is advantageous to animal-based surveillance systems, as it has been amply shown that inferred TBEV prevalence based on ticks removed from hosts is higher compared to questing ticks [14,34,35]. Furthermore, an experimental study revealed that TBEV replication is faster in feeding ticks, resulting in a 500-fold increase in viral load over a 15 h observation period, while in unfed ticks it remains stable [35]. The same study indicated that infected ticks showed highest levels of activity and aggressiveness. All this suggests that animals are likely to amplify the signal of TBEV presence in a given area, which hypothetically increases the chances of successful viral detection.

We believe that our proposed TBEV surveillance technique based on milk sample testing is robust and reliable, in addition to being in good agreement with most of CDC Guidelines for Evaluating Public Health Surveillance Systems recommendations [36]. Generally, ease of operation is one of the key features of our proposed method. This strategy does not require much coordination with numerous institutions or qualified staff. We also believe this method to be more efficient in terms of time, as sampling can rely on volunteer farmers, while the periodic collection of milk could be guaranteed by specialists from local veterinary services. The admissibility criterion, i.e., the willingness of all involved to adopt the method, was also fully met. Most farmers kindly agreed to participate in the study. Many of them showed great interest, upon learning of the investigation, and sought to be included as participants due to concerns about the quality and safety of their products. If the engagement were continuous, paid testing could be considered, especially since it would further allow extension of the flexibility criterion which cannot be considered negligible. Milk testing for TBEV can be integrated into ongoing milk-borne disease monitoring programs or regular milk quality and safety control schemes using currently available infrastructure.

Our proposed method does not allow year-round monitoring, however the period during which small ruminants graze and produce milk coincides perfectly with the seasonal distribution of confirmed TBE cases in humans [37]. Although it appears that the density of small ruminants in Western Europe is much higher than in Lithuania [38], there seem to be some minor shortcomings in applying this method to other countries due to non-homogenous geographic dispersion of farms. In Germany, for example, the majority of small ruminant farms are situated in the southern states [32]. In such cases, the method could at least be adopted for monitoring targeted endemic areas or identifying suspected foci.

Stability and availability are two other features that comply with CDC recommendations. Consumption of unpasteurized milk and related products appears to be on the rise due to alleged health benefits and better taste [18]. A similar trend is observed in the Baltic states. In most of the farms we surveyed, the owners consumed untreated milk products or produced them for sale. As far as farm owners should adapt to market conditions, it is unlikely that this sector will lose its potential applicability to perform a TBEV monitoring program.

Finally, milk sample testing may offer an additional advantage, namely the assessment of milk safety. To date, there have not been many attempts to isolate TBEV from milk. Moreover, such efforts have mostly been implemented in epidemiological analyses of alimentary outbreaks rather than routine molecular screening [39]. Although accurate empirical data and detailed epidemiological studies are not available, it is thought that 7.8% of TBE cases in Lithuania are milk-borne [37]. An even higher fraction was recently observed in Slovakia where up to 17% of TBE cases are caused by alimentary transmission. The relevance of this problem was confirmed by our results showing that nearly one-fifth of positive milk samples were viable to infect cells. In light of high rates of alimentary transmission, periodic milk testing and safety assessment for TBEV, accompanied by public educational activities regarding potential risks of untreated milk product consumption, are greatly needed.

We should also note the gap in fundamental understanding of animal immune responses against TBEV. Based on limited data, animals previously infected with TBE appear to cease shedding the virus into milk [24]. However, it is not clear whether all animals exposed to the virus develop an immune response [40,41]. While the application of our proposed method is certainly not hindered by unstable farm populations that are constantly being replenished by new susceptible animals, further background research would allow for the development of a more precise surveillance strategy.

A number of TBEV monitoring strategies based on serological testing of various vertebrate hosts have recently been developed, many of which show promise [41–46]. However, seroprevalence studies have significant limitations. Antibody persistence rates vary, making it difficult or impossible to predict when an infection occurred. Due to well-known cross-reactivity of TBEV with related flaviviruses [47], serological assessment requires confirmatory assays, which in turn increases the need for more labor and infrastructure, while the interventional nature of studies involving domestic animals requires not only qualified staff and bioethical authorization, but also consent from farm owners, often leading to many farmers refusing to participate in this type of research due to adverse effects on animal productivity.

In conclusion, bulk milk tank samples of small ruminants may serve as a valuable tool for TBEV prevalence analysis and assessment of the epidemiological situation. Because the technique we propose is reliable, non-invasive and easy-to-operate, it may be considered for national surveillance or adapted for monitoring endemic areas and complement human TBE surveillance efforts.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/v13091772/s1, Table S1: Milk amount per bulk tank in tested farms, Figure S1: Phylogenetic tree of the obtained TBEV sequences based on NCR genome fragment.

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References

1. Süss, J. Tick-borne encephalitis 2010: Epidemiology, risk areas, and virus strains in Europe and Asia—An overview. *Ticks Tick-Borne Dis.* **2011**, *2*, 2–15. [CrossRef]
2. Varlacher, J.-F.; Hägglund, S.; Juremalm, M.; Blomqvist, G.; Renström, L.; Zohari, S.; Leijon, M.; Chirico, J. Tick-borne encephalitis: -EN- -FR- Encéphalite transmise par les tiques -ES- Encefalitis transmitida por garrapatas. *Rev. Sci. Tech. OIE* **2015**, *34*, 453–466. [CrossRef]
3. Ilic, M.; Barbic, L.; Bogdanic, M.; Tabain, I.; Savic, V.; Licina, M.L.K.; Kaic, B.; Jungic, A.; Vucelja, M.; Angelov, V.; et al. Tick-borne encephalitis outbreak following raw goat milk consumption in a new micro-location, Croatia, June 2019. *Ticks Tick-Borne Dis.* **2020**, *11*, 101513. [CrossRef]
4. Caini, S.; Szomor, K.; Ferenczi, E.; Gáspár, Á.S.; Csohán, Á.; Krisztalovics, K.; Molnár, Z.; Horváth, J.K. Tick-borne encephalitis transmitted by unpasteurised cow milk in western Hungary, September to October 2011. *Eurosurveillance* **2012**, *17*, 20128. [CrossRef]
5. Holzmann, H.; Aberle, S.W.; Stiasny, K.; Werner, P.; Mischak, A.; Zainer, B.; Netzer, M.; Koppi, S.; Bechter, E.; Heinz, F.X. Tick-borne Encephalitis from Eating Goat Cheese in a Mountain Region of Austria. *Emerg. Infect. Dis.* **2009**, *15*, 1671–1673. [CrossRef] [PubMed]
6. Hudopisk, N.; Korva, M.; Janet, E.; Simetinger, M.; Grgič-Vitek, M.; Gubensek, J.; Natek, V.; Kraigher, A.; Strle, F.; Avšič-Županc, T. Tick-borne Encephalitis Associated with Consumption of Raw Goat Milk, Slovenia, 2012. *Emerg. Infect. Dis.* **2013**, *19*, 806–808. [CrossRef]
7. Markovinović, L.; Kosanović Ličina, M.L.; Tešić, V.; Vojvodić, D.; Vladušić Lucić, I.; Kniewald, T.; Kutleša, M.; Krajinović, L.C. An outbreak of tick-borne encephalitis associated with raw goat milk and cheese consumption, Croatia, 2015. *Infection* **2016**, *44*, 661–665. [CrossRef] [PubMed]
8. Walter, M.; Vogelgesang, J.R.; Rubel, F.; Brugger, K. Tick-Borne Encephalitis Virus and Its European Distribution in Ticks and En-dothermic Mammals. *Microorganisms* **2020**, *8*, 1065. [CrossRef] [PubMed]
9. Erber, W.; Schmitt, H.-J. Self-reported tick-borne encephalitis (TBE) vaccination coverage in Europe: Results from a cross-sectional study. *Ticks Tick-Borne Dis.* **2018**, *9*, 768–777. [CrossRef] [PubMed]
10. Süss, J. Tick-borne encephalitis in Europe and beyond—The epidemiological situation as of 2007. *Eurosurveillance* **2008**, *13*, 18916. [CrossRef] [PubMed]
11. Daniel, M.; Kříž, B.; Danielová, V.; Valter, J.; Kott, I. Correlation between meteorological factors and tick-borne encephalitis inci-dence in the Czech Republic. *Parasitol. Res.* **2008**, *103*, 97–107. [CrossRef]
12. Randolph, S.E. Tick-borne encephalitis incidence in Central and Eastern Europe: Consequences of political transition. *Microbes Infect.* **2008**, *10*, 209–216. [CrossRef]
13. Randolph, S.E. Collective on behalf of the EDEN-TBD sub-project team Human activities predominate in determining changing incidence of tick-borne encephalitis in Europe. *Eurosurveillance* **2010**, *15*, 19606–19631. [CrossRef] [PubMed]
14. Süss, J.; Schrader, C.; Falk, U.; Wohanka, N. Tick-borne encephalitis (TBE) in Germany—Epidemiological data, development of risk areas and virus prevalence in field-collected ticks and in ticks removed from humans. *Int. J. Med Microbiol. Suppl.* **2004**, *293*, 69–79. [CrossRef]
15. Stefanoff, P.; Pfeffer, M.; Hellenbrand, W.; Rogalska, J.; Rühe, F.; Makówka, A.; Michalik, J.; Wodecka, B.; Rymaszewska, A.; Kiewra, D. Virus Detection in Questing Ticks is not a Sen-sitive Indicator for Risk Assessment of Tick-Borne Encephalitis in Humans: TBEV Detection in Ticks to Assess TBE Risk. *Zo-Onoses Public Health* **2013**, *60*, 215–226. [CrossRef] [PubMed]
16. Blomqvist, G.; Näslund, K.; Svensson, L.; Beck, C.; Valarcher, J.F. Mapping geographical areas at risk for tick-borne encephalitis (TBE) by analysing bulk tank milk from Swedish dairy cattle herds for the presence of TBE virus–specific antibodies. *Acta Veter-Scand.* **2021**, *63*, 1–9. [CrossRef]
17. Wallenhammar, A.; Lindqvist, R.; Asghar, N.; Gunaltay, S.; Fredlund, H.; Davidsson, Å.; Andersson, S.; Överby, A.K.; Johansson, M. Revealing new tick-borne encephali-tis virus foci by screening antibodies in sheep milk. *Parasites Vectors* **2020**, *13*, 185. [CrossRef] [PubMed]
18. Paulsen, K.M.; Stuen, S.; das Neves, C.G.; Suhel, F.; Gurung, D.; Soleng, A.; Stiasny, K.; Vikse, R.; Andreassen, Å.K.; Granquist, E.G. Tick-borne encephalitis virus in cows and unpas-teurized cow milk from Norway. *Zoonoses Public Health* **2019**, *66*, 216–222. [CrossRef] [PubMed]
19. Cisak, E.; Wójcik-Fatla, A.; Zając, 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. *Ann. Agric. Environ. Med.* **2010**, *17*, 283–286.
20. 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]

21. 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**, *286*, 125–138. [CrossRef]
22. Van Tongeren, H.A. Encephalitis in Austria. IV. Excretion of virus by milk of the experimentally infected goat. *Arch Gesamte Virusforsch* **1955**, *6*, 158–162. [PubMed]
23. Gritsun, T.S.; Lashkevich, V.A.; Gould, E.A. Tick-borne encephalitis. *Antivir. Res.* **2003**, *57*, 129–146. [CrossRef]
24. Balogh, Z.; Egyed, L.; Ferenczi, E.; Bán, E.; Szomor, K.N.; Takács, M.; Berencsi, G. Experimental Infection of Goats with Tick-Borne Enceph-alitis Virus and the Possibilities to Prevent Virus Transmission by Raw Goat Milk. *Intervirology* **2012**, *55*, 194–200. [CrossRef] [PubMed]
25. 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 Diseases* **2021**, *12*, 101594. [CrossRef] [PubMed]
26. Rieille, N.; Bressanelli, S.; Freire, C.C.M.; Arcioni, S.; Gern, L.; Péter, O.; Voordouw, M.J. Prevalence and phylogenetic analysis of tick-borne en-cephalitis virus (TBEV) in field-collected ticks (Ixodes ricinus) in southern Switzerland. *Parasit Vectors* **2014**, *7*, 443. [CrossRef] [PubMed]
27. Lommano, E.; Burri, C.; Maeder, G.; Guerne, M.; Bastic, V.; Patalas, E.; Gern, L. Prevalence and Genotyping of Tick-Borne Encephalitis Virus in QuestingIxodes ricinusTicks in a New Endemic Area in Western Switzerland. *J. Med. Ѐntomol.* **2012**, *49*, 156–164. [CrossRef] [PubMed]
28. 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 in-cidence in 2006 independent of variable tick abundance but related to weather. *Parasites Vectors* **2008**, *1*, 44. [CrossRef]
29. Bormane, A.; Lucenko, I.; Duks, A.; Mavtchoutko, V.; Ranka, R.; Salmina, K.; Baumanis, V. Vectors of tick-borne diseases and epidemiologi-cal situation in latvia in 1993–2002. *Int. J. Med Microbiol. Suppl.* **2004**, *293*, 36–47.
30. Oehme, R.; Hartelt, K.; Backe, H.; Brockmann, S.; Kimmig, P. Foci of tick-borne diseases in Southwest Germany. *Int. J. Med Microbiol.* **2002**, *291*, 22–29. [CrossRef]
31. Gäumann, R.; Mühlemann, K.; Strasser, M.; Beuret, C.M. High-Throughput Procedure for Tick Surveys of Tick-Borne Encephalitis Virus and Its Application in a National Surveillance Study in Switzerland. *Appl. Environ. Microbiol.* **2010**, *76*, 4241–4249. [CrossRef]
32. 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]
33. Piesman, J.; Eisen, L. Prevention of Tick-Borne Diseases. *Annu. Rev. Ѐntomol.* **2008**, *53*, 323–343. [CrossRef]
34. 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. *Pathog* **2021**, *10*, 140. [CrossRef]
35. Belova, O.A.; Burenkova, L.A.; Karganova, G.G. Different tick-borne encephalitis virus (TBEV) prevalences in unfed versus par-tially engorged ixodid ticks—Evidence of virus replication and changes in tick behavior. *Ticks Tick-Borne Dis.* **2012**, *3*, 240–246. [CrossRef]
36. Updated Guidelines for Evaluating Public Health Surveillance Systems; Recommendations from the Guidelines Working Group. *Centers for Disease Control and Prevention. v. 50, no. RR-13*; 2001. Available online: https://stacks.cdc.gov/view/cdc/13376 (accessed on 1 September 2021).
37. 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 retro-spective study. *Eur. J. Neurol.* **2018**, *25*, 268–274. [CrossRef] [PubMed]
38. Gilbert, M.; Nicolas, G.; Cinardi, G.; Van Boeckel, T.P.; Vanwambeke, S.; Wint, G.R.W.; Robinson, T.P. Global distribution data for cattle, buffaloes, horses, sheep, goats, pigs, chickens and ducks in 2010. *Sci. Data* **2018**, *5*, 180227. [CrossRef]
39. Brockmann, S.; Oehme, R.; Buckenmaier, T.; Beer, M.; Jeffery-Smith, A.; Spannenkrebs, M.; Haag-Milz, S.; Wagner-Wiening, C.; Schlegel, C.; Fritz, J.; et al. A cluster of two human cases of tick-borne encephalitis (TBE) transmitted by unpasteurised goat milk and cheese in Germany, May 2016. *Eurosurveillance* **2018**, *23*. [CrossRef] [PubMed]
40. Klaus, C.; Hoffmann, B.; Moog, U.; Schau, U.; Beer, M.; Süss, J. Can goats be used as sentinels for tick-borne encephalitis (TBE) in nonendemic areas? Experimental studies and epizootiological observations. *Berl. Munch. Tierarztl. Wochenschr.* **2010**, *123*, 441–445. [PubMed]
41. Klaus, C.; Beer, M.; Saier, R.; Schau, U.; Moog, U.; Hoffmann, B.; Diller, R.; Süss, J. 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 Tick-Borne Dis.* **2012**, *3*, 27–37. [CrossRef]
42. Rizzoli, A.; Neteler, M.; Rosa, R.; Versini, W.; Cristofolini, A.; Bregoli, M.; Buckley, A.; Gould, E.A. Early detection of tick-borne encephalitis virus spatial distribution and activity in the province of Trento, northern Italy. *Geospat. Heal.* **2007**, *1*, 169. [CrossRef] [PubMed]
43. Skarphedinsson, S.; Jensen, P.M.; Kristiansen, K. Survey of Tickborne Infections in Denmark. *Emerg. Infect. Dis.* **2005**, *11*, 1055–1061. [CrossRef] [PubMed]
44. Wurm, R.; Dobler, G.; Peters, M.; Kiessig, S.T. Serological Investigations of Red Foxes (*Vulpes vulpes* L.) for Determination of the Spread of Tick-borne Encephalitis in Northrhine-Westphalia. *J. Vet. Med. Ser. B* **2000**, *47*, 503–509. [CrossRef] [PubMed]

45. Burri, C.; Korva, M.; Bastic, V.; Knap, N.; Avsic-Zupanc, T.; Gern, L. Serological evidence of tick-borne encephalitis virus infection in rodents captured at four sites in Switzerland. *J. Med. Èntomol.* **2012**, *49*, 436–439. [CrossRef]
46. Lindhe, K.E.; Meldgaard, D.S.; Jensen, P.M.; A Houser, G.; Berendt, M. Prevalence of tick-borne encephalitis virus antibodies in dogs from Denmark. *Acta Vet. Scand.* **2009**, *51*, 56. [CrossRef]
47. 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*, 78. [CrossRef] [PubMed]

Article

Cross-Sectional Study on the Prevalence and Factors Influencing Occurrence of Tick-Borne Encephalitis in Horses in Lithuania

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Abstract: Various animal species have been evaluated in depth for their potential as Tick-borne encephalitis virus (TBEV) sentinel species, although evidence for equine capacity is incomplete. Therefore, a comprehensive cross-sectional stratified serosurvey and PCR analysis of selected horses (n = 301) were performed in TBEV endemic localities in Lithuania. Attached and moving ticks (n = 241) have been collected from aforementioned hosts to evaluate natural infectivity of TBEV vectors (*Ixodes spp.*) in the recreational environments surrounding equestrian centers. All samples were screened for TBEV IgG and positive samples were confirmed by virus neutralization test (VNT). 113 (37.5%) horses from all counties of Lithuania tested positive for TBEV IgG, revealing age and sex indifferent results of equine seroprevalence that were significantly dependent on pedigree: horses of mixed breed were more susceptible to infection possibly due to their management practices. TBEV prevalence in equine species corresponded to TBEV-confirmed human cases in the precedent year. As much as 3.9% of horses were viraemic with TBEV-RNA with subsequent confirmation of TBEV European subtype. 4/38 of tested tick pools were positive for TBEV-RNA (Minimal infectious rate 1.2%). Several unknown microfoci were revealed during the study indicating areas of extreme risk close to popular human entertainment sites. The study provides important evidence in favor of horses' usage as sentinel species, as equines could provide more detailed epidemiological mapping of TBEV, as well as more efficient collection of ticks for surveillance studies.

Keywords: TBE; TBEV; tick-borne encephalitis; TBE seroprevalence

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1. Introduction

Tick-borne encephalitis (TBE) is the most important tick-borne viral zoonotic disease in Europe caused by a bite of TBE virus (Flavivirus, Flaviviridae) infected *Ixodes* spp. [1]. In Lithuania TBE incidence rates have been increasing by 8.5% per year for the 45-year

period from 1970 to 2014 [2]. Moreover, the country has the highest incidence rate of the disease in Europe since 2013 [3].

Tick-borne encephalitis is endemic throughout Lithuania [4]. The number of autochthonous confirmed clinical TBE cases with known site of exposure define a risk level for TBE in the region. Since the actual population at risk can deviate greatly from the number of inhabitants in an area due to the focal distribution of TBE, risk estimates based on incidence have limitations, especially if large geographic areas are chosen [5]. In addition, socioeconomic factors, preventive measures such as high vaccine coverage and recreational usage of a natural area can significantly alter results of disease monitoring, necessitating alternative methods to define the degree of risk and endemicity.

Wild and domestic animals have already raised an interest as surrogate markers of natural Tick-borne encephalitis virus (TBEV) prevalence [6–8]. Significant correlation between seroprevalence in small mammals, dogs, bovids, cervids and TBE incidence in humans was confirmed in known endemic areas [9–13], as well as the capacity of these sentinel species to uncover presently unknown TBEV foci, whereas insights to equine contribution to wild TBEV dynamics are lacking [6].

Clinical TBE cases in veterinary medicine are rare but can manifest with varying degree of non-specific neurological symptoms. In horses, reduced general condition, behavioral changes, ataxia and paralysis of neck and shoulder muscles have been described [14]. Laboratory diagnosis is essential for TBE confirmation, such as detection of TBEV IgM and/or TBEV IgG antibodies in serum or cerebrospinal fluid by immunoassays. Due to well known serological cross-reactivity with other flaviviruses, case validation requires subsequent confirmation of all enzyme-linked immunosorbent assay (ELISA) positive results by virus neutralization test (VNT) or PCR [14].

Horses have scarcely been investigated as potential TBEV sentinel species and only a few articles describe the epidemiological role of equines for TBEV circulation in nature [14–16]. Therefore, to evaluate equine contribution to TBEV and sentinel-species capacity we report a cross-sectional study performed on the prevalence and factors influencing occurrence of tick-borne encephalitis in horses in Lithuania.

2. Materials and Methods

2.1. Sample Collection and Sampling Sites

In order to accurately represent a cross-sectional study design that analyzes data collected at one given point in time [17], equine blood samples (n = 301) were collected in one month (May 2019), at the peak of first tick questing period. Stables were added to the study if they adhered to the inclusion criteria: sampled horses were turned out in pastures every day, had a well defined territorial radius and were not vaccinated for any flaviviruses.

After applying stratified random sample approach, 32 equestrian centers were examined (Table 1). Each stratum of equine blood samples in our study was estimated to represent equine population in each of the counties of Lithuania with a 5% margin of error (CI 95%) based on the national database provided by National Land Service under the Ministry of Agriculture of the Republic of Lithuania. To assess factors of influence to occurrence of the TBE, detailed information was collected regarding stable-level risk factors such as herd size, their ration composition, daily pasture time and animal-level risk factors: age, breed, gender of the horses, prior travel and health records.

Blood was drawn with regard to animal welfare regulations [18], then serum samples of 3–4 mL in volume were separated by double centrifugation process, transferred to 2 mL tubes and stored at −20 °C until further use.

In addition, attached or moving ticks (n = 241) were collected from aforementioned hosts where possible. Pools were formed depending on the number of ticks collected from a given location, 5–10 nymphs or 2–5 adult ticks per pool. The ticks were dissected and homogenized in phosphate-buffered saline (PBS; 1×, pH 7.2; Gibco, Grand Island, NY, USA), inserted

into liquid nitrogen and then ground into a fine powder in a mortar. Each homogenized suspension was centrifuged, supernatant was collected and stored at −20 °C.

Table 1. Results of equine serology and PCR of serum and ticks in 32 tested stables.

No. of the Stable	Sample Size of the Stable	County	Seroprevalence in the County	TBEV Seropositive Horses	Seroprevalence in the Stables	CI 95 %	PCR Positive Serum Samples	Tick Pools	PCR Positive Tick Pools
1	12	Klaipėda	42.3	8	66.6	34.8–90.0	0	4	0
2	14			3	21.4	0.4–50.8	0	2	0
3	12	Telsiai	8.3	1	8.3	0.2–38.4	0	0	0
4	7	Siauliai	30.6	4	57.1	18.4–90.1	1	1	0
5	10			1	10.0	0.2–44.5	3	0	0
6	19			6	31.5	12.5–56.5	0	0	0
7	12	Panevezys	46.2	5	41.6	15.1–72.3	1	0	0
8	14			7	51.0	23.0–76.9	1	0	0
9	13	Utena	30.8	4	30.7	0.9–61.4	0	0	0
10	3			1	33.3	0.8–90.5	0	0	0
11	4			1	25.0	0.6–80.5	0	0	0
12	11			4	36.3	10.9–69.2	0	3	0
13	15			5	33.3	11.8–61.6	1	2	0
14	6			1	16.6	0.4–64.1	0	0	0
15	8	Vilnius	50.6	1	12.5	0.3–52.6	0	1	0
16	6			6	100.0	54.0–100	0	1	0
17	2			1	50.0	0.1–98.7	0	0	0
18	14			14	100.0	0.7–100	0	22	4
19	17			9	52.9	27.8–77.0	0	0	0
20	17			2	11.7	0.1–36.4	2	0	0
21	17			7	41.1	18.4–67.0	0	0	0
22	6			4	66.6	22.2–95.6	0	0	0
23	2	Marijampole	22.2	2	100.0	15.8–100.0	0	0	0
24	7			0	0.0	0–40.9	0	0	0
25	4	Kaunas	34.2	4	100.0	39.7–100.0	0	1	0
26	6			2	33.3	0.4–77.7	0	0	0
27	8			2	25.0	3.1–65.0	0	0	0
28	13			5	38.4	13.8–68.4	0	0	0
29	1			0	0.0	0.0–97.5	0	0	0
30	6			0	0.0	0.0–45.9	0	0	0
31	5	Alytus	20.0	2	40.0	0.5–85.3	0	0	0
32	10			1	10.0	0.2–44.5	3	1	0
Total	301			113	37.5	32.3–43.1	12	38	4

2.2. Data of Human TBEV Cases

Data concerning confirmed human TBEV cases in different counties of Lithuania were obtained from the Centre for Communicable Diseases and AIDS of Lithuania and population data for the calculation of TBE seroprevalence were obtained from the Lithuanian Department of Statistics.

2.3. ELISA and Virus Neutralization Test

The serum samples were tested by ELISA for TBEV IgG using the EIA TBEV IgG kit (TestLine Clinical Diagnostics, Brno-Královo Pole, Czech Republic) following manufacturer's protocol. The results were calculated as the negative control/sample ratio, and a < 150% was used as a cutoff value for negative samples, 200%> for positive samples. All serum samples were retested by VNT for case confirmation and exclusion of serological cross-reactivities with other flaviviruses. The TBEV-specific neutralizing antibodies were determined using gold standard in-house neutralization assay [19]. Prior to testing, horse serum samples were complement inactivated and diluted starting from 1/5 to 1/320 in Minimum Essential Medium (MEM) (Gibco, Grand Island, NY, USA). The serum dilutions were incubated with 100 $TCID_{50}$ of TBEV strain obtained after 10 passages of

cultivation on Vero cell culture (ATCC® CCL-81™, Manassas, VA, USA). Cells were assessed for the presence of cytopathogenic effects at 3, 5 and 7 days post-infection (p. i.). TBEV reciprocal titre of $\geq 1/20$ was considered as positive in VNT.

Due to discrepancy of positive samples yielded after serology and VNT, we have raised a concern of possible concomitant circulation of multiple flaviviruses in Lithuania and performed an additional ELISA test for West Nile virus IgG using WN Competition Multi-species Ig kit (ID Screen, ID.vet, Grabels, France).

2.4. TBE Virus Detection and Viral Load Quantification

Total RNA was extracted from 300 µL serum and tick suspension samples using the GeneJET RNA Purification Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Samples were screened by conventional and real time reverse transcription PCR for the presence of specific TBEV RNA using primer sets described previously [20,21]. Reaction mix SuperScript™ III One-Step RT-PCR System with Platinum™ *Taq* DNA Polymerase (Thermo Scientific, Waltham, MA, USA) was utilized for real time PCR and DreamTaq Green PCR Master Mix (2×) (Thermo Scientific, Waltham, MA, USA) for conventional PCR. PCR reactions were carried out in triplicates.

We modified viral quantification assay as it was previously described [20]. Briefly, a synthetic fragment corresponding to the amplified region of the TBEV was cloned into the pJET1.2 vector using the CloneJET PCR Cloning Kit (Thermo Scientific, Waltham, MA, USA) and Transform-Aid Bacterial Transformation Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Following transformation of *E. coli* cells, plasmid DNA extraction and purification was performed using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, Waltham, MA, USA) according to supplier's protocol. Standard curves were generated after 10-fold dilutions of stock DNA (supplementary material Figure S1). Reactions were carried out in triplicates.

Quality of RNA extraction of tick samples was assessed using RT-qPCR targeting 16s rRNA of *I.ricinus* as described elsewhere [20].

2.5. Virus Isolation

Virus was isolated in Vero (ATCC® CCL-81™, Manassas, VA, USA) cell line and serial passages were performed to assess its viability to infect cells and obtain sufficient number of viral copies necessary for sequencing.

Samples were passed through a 0.22-µm pore size microfilter (Techno Plastic Products AG, Trasadingen, Switzerland) for purification. Cells were then inoculated in 25 cm^2 tissue culture flasks (TPP Techno Plastic Products AG, Trasadingen, Switzerland) for 1h at 37 °C. Negative controls were inoculated with MEM and 100 U mL^{-1} penicillin and 100 µg L^{-1} streptomycin only. The inoculate was removed and 10 mL of Minimum Essential Medium with additional 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) was added. Cells were incubated at 37 °C in 5% CO_2 and air mixture and was examined for the occurrence of cytopathic effect through 5 serial passages which were performed in triplicate set frame including triplicate of positive and negative controls for each round of analysis.

2.6. Sequencing and Phylogenetic Analysis

For further genetic characterization and positive sample confirmation partial genome sequencing was performed. Multiple alignment of all sequences was created using ClustalW software (Clustal, Dublin, Ireland) in MEGA X package. The neighbor-joining method was used for phylogenetic tree construction with 1000 bootstrapping replicates. Sequences of different TBEV strains and closely related flaviviruses chosen from the NCBI GenBank database were used for phylogenetic comparisons.

2.7. Statistical Analysis

Confidence intervals of seroprevalence were based on the exact binomial method. Binary logistic regression analysis was used to test the significance of the differences and odds ratio (OR), regression coefficient (β), and standard error (SE) in age-, sex- and breed-specific antibody prevalence. Chi-square test was used to calculate associations between pasture time and seropositivity. Pearson correlation was used to evaluate relation of human and horse TBEV cases. The minimal infection rate (MIR) was calculated as the ratio of the number of positive pools to the total number of ticks tested. MIR = number of positive pools/total number of ticks tested × 100%. Results with p value were regarded as significant. All statistical analysis and mapping was performed using the programming language R-project (4.03).

3. Results

3.1. ELISA and Neutralization Assay

Based on ELISA assay, 124 (41.2%), 5 (1.7%) and 172 (57.1%) serum samples were considered positive, borderline and negative for the presence of TBEV-specific antibodies, respectively. Thus, optical density plot showed clear bimodal distribution (Figure 1A). All samples were retested by virus neutralization assay. ELISA negative samples were consistent with VNT; however, only 109 and 4 samples were confirmed as positive in ELISA-positive and ELISA-borderline groups, respectively. Therefore, for further analysis, the overall number of TBE seropositive samples was adjusted according to VNT results at 113 (37.5%; 95% CI 32.2–43.1).

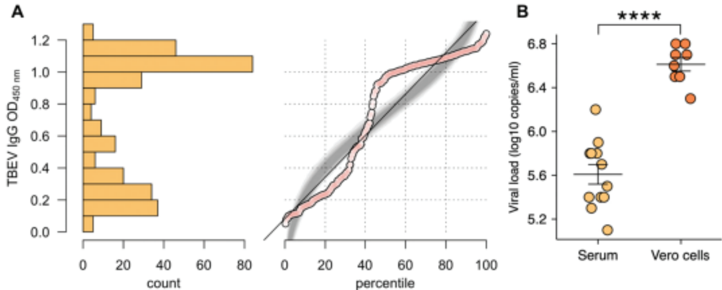

Figure 1. (**A**) Normal quantile plot of the distribution of optical density (OD) 450 nm values in Tick-borne encephalitis virus (TBEV) immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) with sera collected from horses. Percentile plot with simulation (grey) of a normal distribution with the same mean and standard deviation as for the data collected; (**B**) Difference in TBE viral loads between cultured medium and serum-originated samples. (**** $p < 0.0001$).

The study comprised 145 (48.2%) mares, 20 (6.6%) stallions and 136 (45.2%) geldings. No statistically significant associations were found between ration composition, herd size, age, gender and seropositivity.

Horses investigated in our study consisted of 26 breeds, as well as mixed breed individuals that represented almost a third of all tested equids (n = 85; 28.2%). Binary logistic regression model revealed significant association between the pedigree and serological results: seroprevalence of 31.0% (95% CI 24.9–37.6) and 53.4% (95% CI 42.41–64.3) of horses with known origin and mix-breeds, respectively ($p < 0.03$; OR 2.3; CI 95% 1.3–4.0; β 0.8; SE 0.3). In addition, we have found a significant relation between daily time spent in the pastures and seropositivity as horses that spent >8 h in the field had more than 2 times higher seroprevalence (68.0%; CI 95% 59.8–75.4) than horses that spent <8 h daily (31.7%; CI 95% 18.0–48.0). Notwithstanding the obvious difference, endmost result should be

assessed ambiguously as the data was collected through a questionnaire survey of animal owners which may be affected by undefined bias.

Concerning equine sera tested with ELISA for detection of West Nile virus antibodies, we have found that some horses showed WNV seropositivity and negative TBEV VNT with 100 $TCID_{50}$ of TBEV strain (data not shown).

Analysis of horse medical records showed no association with TBEV seropositivity. 11 horses demonstrated certain degree of atypical behavior or balance disturbances that we attributed to underdiagnosed cases of non-infectious origin, even though five of them were positive for TBEV neutralizing antibodies. Travel history of 17 horses included neighboring countries, although most of these equines did not travel outside Lithuania in the past 2 years at the time of sampling.

3.2. Virus Detection and Isolation

Due to a short time viremia, after which flaviviruses are cleared from serum, direct virus detection is limited in large herbivores and to our best knowledge we are first to report successful PCR and sequencing results in horses. As many as 12 (3.9%; 95% CI 2.3–6.8) of all tested equine serum samples were confirmed positive for the TBE virus. In addition, 4/38 of tested tick pools were positive for TBEV-RNA (MIR 1.2%) All positive tick samples were in adult tick stage of *I. ricinus* obtained from the same stable.

Presence of infective virus in serum samples and tick homogenates was confirmed by PCR analysis of the Vero cell culture supernatants collected at 5–6 days p.i. in all tested tick samples and 8 horse serum samples. Load of virus particles was assessed at different time points. Titres shown in this paper were assessed after the fourth passage at the load peak (Figure 1B)

3.3. Sequencing

Successful virus isolation was confirmed by partial genome sequencing based on NS5 fragment of TBEV isolates retrieved from cell cultures (Figure 2). Sequence data have been submitted to GenBank database under Accession Numbers MT981174-MT981178 and MW187721-MW187725. BLASTN search (NCBI Genbank) revealed that all DNA sequences of equine serum in this study were 93.0–97.1% similar to the reference strain of the European subtype (Neudörfl; Genbank U27495). In contrast, the similarity with the reference strains of the Far Eastern subtype (Sofjin; AB062064) and the Siberian subtype (Vasilchenko; AF069066) were 78.3 and 84.4%, respectively. TBEV sequences obtained from ticks in the present study shared a high degree of similarity (99.6%) to European subtype.

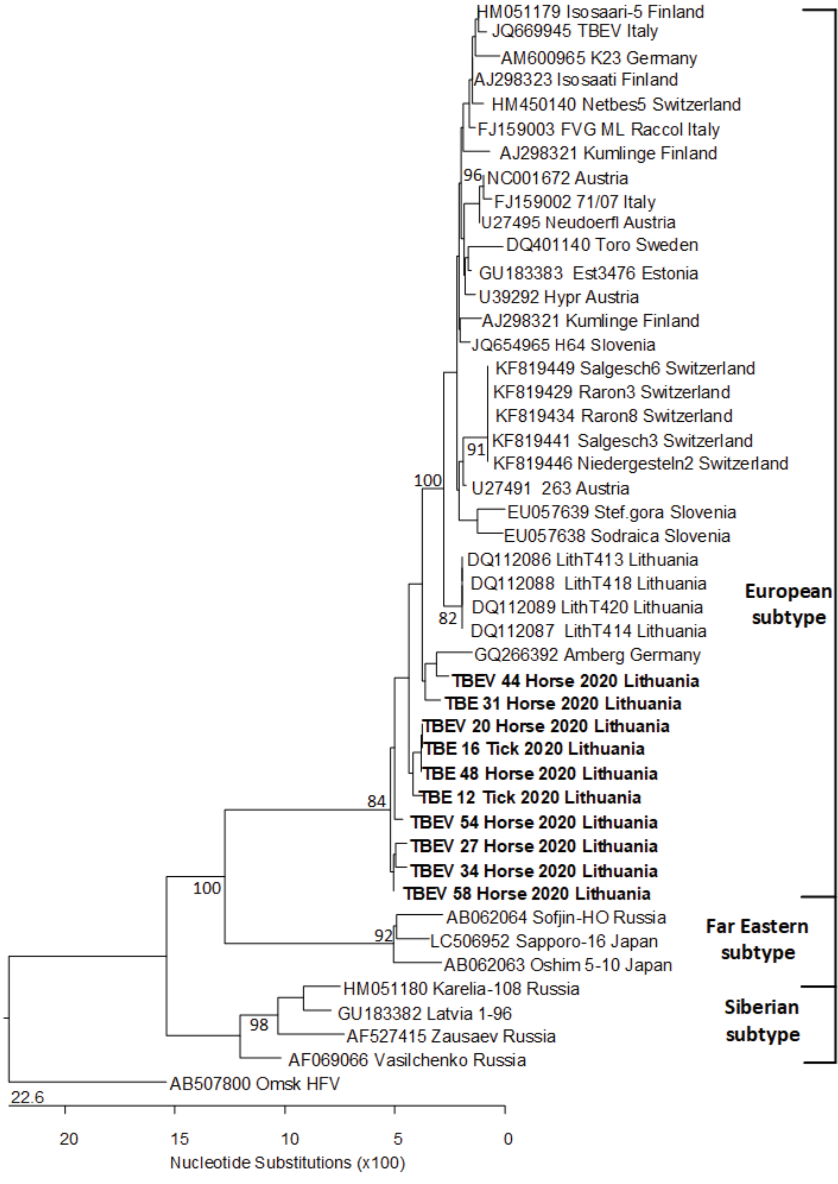

Figure 2. Phylogenetic tree of the TBEV sequences based on NS5 gene. The Omsk hemorrhagic fever virus was used as an outgroup. The sequences obtained in our study are shown in bold. Sequence data have been submitted to GenBank database under Accession Numbers MT981174-MT981178 and MW187721-MW187725.

3.4. Spatial Distribution and of TBEV Specific Antibodies

Spatial pattern corresponding to the sampling locations is shown in Figure 3. To ensure anonymization and personal data protection, the geographic coordinates reflecting the exact location of the stables were randomly shifted to one of the cardinal directions in the range of 10 to 50 km without overstepping the stratum of sampling.

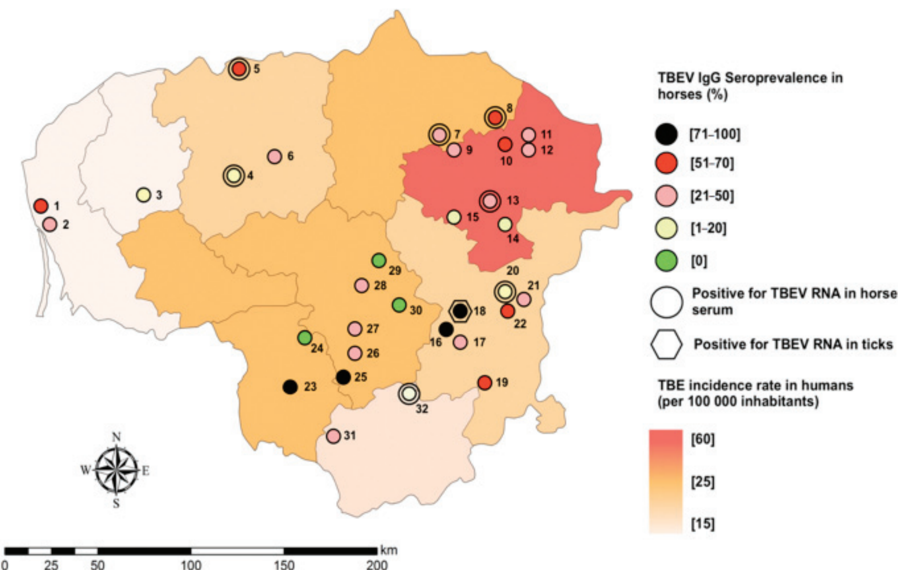

Figure 3. Spatial distribution of TBEV RNA and TBEV-neutralizing IgG seroprevalence at the county level in Lithuania. The colour grades represent the incidence rate in human TBE infection in 2019. Locations of sample acquisition are identified in numbers that are represented in Table 1. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

TBEV neutralizing antibodies were detected in 29 (90.6%, 95% CI 74.9–98.0) of all tested stables covering all counties involved in the study (Table 1).

Spatial analysis showed statistically significant differences in average seroprevalence amongst counties of the country. Highest seroprevalence rates were observed in east part of Lithuania with overall highest seroprevalence in Vilnius (50.6%; CI 95% 39.6–61.1) county ($p < 0.04$; OR 3.3; CI 95% 1.0–10.7; β 1.2; SE 0.6).

In the study year, insignificant positive correlation between TBEV seropositivity in tested horses and TBE-incidence rate in humans in given administrative unit was observed (r = 0.29). In contrast, strong positive correlation between these variables were detected with human TBE incidence of 2018 (r = 0.76; $p < 0.05$).

4. Discussion

Our study provides a first comprehensive investigation into prevalence of TBEV in equines in the northern Europe. We have revealed significantly higher seroprevalence of TBEV in horses (37.5% (95% CI 32.2–43.1) than previously recorded in Lithuania where in all domestic animal species tested TBE seropositivity of only 8.6% in 2003 and 1.7% in 2005 was found with considerable regional differences. Immense differences of results may be due to the strict inclusion criteria of sampling that allowed only horses with plausible

exposure to TBEV infection to be examined e.g., sport horses that are ridden indoors were not included in the study. Interestingly, this upsurge of TBEV seroprevalence corresponds to the pattern observed in humans where a joinpoint analysis revealed 7.4% annual increase of TBEV incidence rate in 2005–2014 [2] in spite of characteristic fluctuations of TBE cases observed between tick questing seasons [22–24].

Contrary to the tendency observed by Rushton et al. [15], our study did not find significant associations between sex of the horse and seropositivity. This result may be influenced by different management conditions imposed on stallions in Austria [15], whereas in our study, stallions were mostly kept in comparable environments to those of mares. In addition to this, high tick density and infectivity in Lithuania may be underlying factor for non-differentiating results in both sexes.

In our study, age of the horses was an insignificant factor to TBEV infection unlike in equines in Austria where younger animals seemed to be more prone to infection [15] and previous studies of cattle, where animals up to 3 years old had significantly lower seroprevalence [25].

After having tested foals, we have found agreeing evidence that maternal immunity could be passed via colostrum, as most foals under 7 months old were seropositive with considerably high antibody values. Yearlings showed more varying results as an indication to fading antibodies of passive transfer.

One of the TBEV prevalence defining factor in our study was breed and more specifically, pedigree. As much as it can be tempting to rely on genetics, we think that this connection is purely based on the difference in management and purpose of mixed and purebred horses. Horses without defined pedigree are more likely to be used for long hikes in nature, as well as leisure and tourism. In addition, they were kept in pastures for significantly longer periods of time; therefore, they had higher probability of contracting a tick-borne infection.

Virus detection and sequencing processes revealed that as much as 3.9% (95% CI 2.3–6.8) of tested horses were positive for TBEV-RNA with subsequent confirmation of TBEV European subtype. To our best knowledge, the only study reporting sole sample potentially positive by PCR was performed in Germany. However, their sequencing or cultivation attempt was not successful [14]. Interestingly, the sequences we obtained did not cluster with other Lithuanian sequences. This can be explained by a lack of NS5 viral genome fragment-targeted or whole-genome sequences obtained in given territories as most of studies confirm specific product based on E or NS3 encoding fragments. Thus, further studies on whole genomic sequencing are necessary to get deeper insight on regional genetic diversity and lineages of TBEV strains.

Field collected ticks are known to be time and labor consuming indicators of infection risk [26,27], but it seems that infectivity of ticks is relatively higher, if samples are collected in the vicinity of domestic animals [14]. Our study revealed a relatively high prevalence of TBEV in ticks compared to a nationwide study conducted in Lithuania in 2017–2019 where MIR in field collected ticks was only 0.4% [28]. Almost 3-fold higher minimal infectious rate in ticks is most likely due to sampling properties: in our study ticks were collected from animals where favorable feeding conditions were guaranteed. The latter factor is associated with changes in dynamics of virus reproduction. It has been experimentally proven that intensive viral replication commences during feeding of ticks resulting in 500 times higher viral load in 15h period while in unfed ticks TBEV concentration remains stable [29]. In contrast, aforementioned study was based on sampling of questing ticks where some of the ticks potentially had low concentration of TBEV which therefore was below detection threshold. In addition, infected ticks are significantly more active and aggressive hence, there is a potentially higher probability of TBEV being found in the host rather than among questing ticks [29].

Important findings in this study seem to be the absolute seroprevalence and high values of TBEV IgG observed in equine sera obtained from several locations indicating a recent infection with TBEV from previously unknown microfoci close to these equestrian

centers. In addition, ticks from one of these stables were found to have an infective TBEV confirmed by PCR and virus isolation. Previous studies have proven that sera of game animals is a reliable tool to reveal natural TBEV circulation [30] and we suggest that equine sera too is an important surrogate marker of areas of extreme risk.

Equine sentinel-species capacity is hard to evaluate. Our study has found significant correlation between equine TBEV seropositivity and human TBE cases for precedent year as equine individuals seem to respond accurately to the grade of infectivity in the geographical areas. On the other hand, seropositive results of horses failed to correspond to human incidence rates on the year of study (2019). We believe these results were biased due to relatively long period of time required for IgG production upon a new TBEV infection and population data discrepancy, as equine samples were collected in May and compared to human incidence rate of complete year in December. Cross-sectional design and population data of large geodemographic areas were important limitations of this study that precluded us from more complete evaluation of TBEV dynamics in horses, although several important aspects for TBEV sentinel species seem to be applicable to equines. Horses are capable to mount their Ab levels after contact with TBEV that could be detectable 9–19 months post-infection [14,31,32]. Interpretation of seroprevalence on the level of the stables could permit mapping of TBEV foci on smaller than district level as high Ab values of positive horses at the equestrian centers indicate recent TBEV exposure. In addition, multiple countries have currently established routine monitoring programs for equine infectious anemia therefore, large sample sizes are readily available. In addition to that, increased MIR of ticks collected from horses roaming large, well defined areas signifies an alternative to labor and time consuming field collection of ticks.

Interestingly, several equine serum samples appeared seropositive for WNV and demonstrated cytopathic effects after VNT with TBEV, indicating first serological evidence of WNV in Lithuania. These results prompt further investigation into possible emergence of new geographical distribution of WNV in northern Europe.

Large herbivores are known as tick mating and feeding sites [33] and could be important in amplifying TBEV vector populations [34], therefore bigger stables could possibly be a reason for increased tick numbers in the area. On the other hand, *Ixodes* tick density do not necessarily correlate with TBEV spread in susceptible species and horses may be held responsible for diverting tick bites from competent hosts, thus diluting pathogen transmission [35]. Pastures and riding trails of horses can theoretically be a suitable medium for TBEV foci as several papers denote the effect of grazing as well as influence of litter layer thickness on tick population [36,37], but continuous multimodal works are required to accurately evaluate the role of horses in the natural spread of the virus.

5. Conclusions

Horses are dead-end hosts of TBEV providing measurable immunity responses upon natural infection with TBEV that seem to accurately represent infectivity of an area. TBEV surveillance studies in horses can reveal new microfoci and permit epidemiological mapping on lower than district level, therefore equines can be attributed as possible TBEV sentinel species.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-0817/10/2/140/s1, Figure S1: Standard curve of qPCR using serial dilutions of stock DNA.

Author Contributions: Conceptualization, A.P., A.A. and A.S. (Arunas Stankevicius); methodology and investigation, A.P., A.A., E.S., R.Z., J.B., J.G., I.Z., A.S. (Algirdas Salomskas) and A.S. (Arunas Stankevicius); data analysis, A.P., A.A. and R.A.-J.; resources, D.Z., A.S. (Arunas Stankevicius), J.B. and A.S. (Algirdas Salomskas); writing—original draft preparation, A.P. and A.A.; writing—review and editing, J.G. and A.S. (Arunas Stankevicius); supervision, A.S. (Arunas Stankevicius); funding acquisition, A.P. and A.S. (Arunas Stankevicius). All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to confidentiality agreements.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Süss, J. Tick-Borne encephalitis 2010: Epidemiology, risk areas, and virus strains in Europe and Asia—An overview. *Ticks Tick Borne Dis.* **2011**, *2*, 2–15. [CrossRef] [PubMed]
2. Radzišauskienė, D.; Žagminas, K.; Ašoklienė, L.; Jasionis, A.; Mameniškienė, R.; Ambrozaitis, A.; Lancoriene, L.; Jatuzis, D.; Petraityte, I.; Mockiene, 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]
3. European Centre for Disease Prevention and Control. *Tick-Borne Encephalitis—Annual Epidemiological Report for 2018*; European Centre for Disease Prevention and Control: Stockholm, Sweden, 2019.
4. European Centre for Disease Prevention and Control. *Country Profile: Lithuania. Tick-Borne Encephalitis (TBE)*; European Centre for Disease Prevention and Control: Stockholm, Sweden, 2012.
5. Lindquist, L. Tick-Borne encephalitis. In *Handbook of Clinical Neurology*; Elsevier Health Sciences: Amsterdam, The Netherlands, 2014; pp. 531–559.
6. 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]
7. Gerth, H.J.; Grimshandl, D.; Stage, B.; Döller, G.; Kunz, C. Roe deer as sentinels for endemicity of tick-borne encephalitis virus. *Epidemiol. Infect.* **1995**, *115*, 355–365. [CrossRef] [PubMed]
8. Achazi, K.; Růžek, D.; Donoso-Mantke, O.; Schlegel, M.; Ali, H.S.; Wenk, M.; Ruhe, F.; Kallies, R.; Vor, T.; Ulrich, R.G.; et al. Rodents as sentinels for the prevalence of tick-borne encephalitis virus. *Vector Borne Zoonotic Dis.* **2011**, *11*, 641–647. [CrossRef] [PubMed]
9. 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]
10. Klaus, C.; Beer, M.; Saier, R.; Schau, U.; Moog, U.; Hoffmann, B.; Diller, R.; Suss, J. 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 Tick Borne Dis.* **2012**, *3*, 27–37. [CrossRef]
11. Klaus, C.; Hoffmann, B.; Beer, M.; Müller, W.; Stark, B.; Bader, W.; Stiasny, K.; Heinz, F.X.; Suss, J. Seroprevalence of tick-borne encephalitis (TBE) in naturally exposed monkeys (*Macaca sylvanus*) and sheep and prevalence of TBE virus in ticks in a TBE endemic area in Germany. *Ticks Tick Borne Dis.* **2010**, *1*, 141–144. [CrossRef]
12. Lindhe, K.E.S.; 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]
13. Duscher, G.G.; Wetscher, M.; Baumgartner, R.; Walder, G. Roe deer sera used for TBE surveillance in Austria. *Ticks Tick Borne Dis.* **2015**, *6*, 489–493. [CrossRef]
14. Klaus, C.; Hörügel, U.; Hoffmann, B.; Beer, M. Tick-Borne encephalitis virus (TBEV) infection in horses: Clinical and laboratory findings and epidemiological investigations. *Vet. Microbiol.* **2013**, *163*, 368–372. [CrossRef] [PubMed]
15. Rushton, J.O.; Lecollinet, S.; Hubálek, Z.; Svobodová, P.; Lussy, H.; Nowotny, N. Tick-Borne Encephalitis Virus in Horses, Austria, 2011. *Emerg. Infect. Dis.* **2013**, *19*, 635–637. [CrossRef] [PubMed]
16. Müller, K.; König, M.; Thiel, H.J. Tick-Borne encephalitis (TBE) with special emphasis on infection in horses. *Dtsch. Tierarztl. Wochenschr.* **2006**, *113*, 147–151. [PubMed]
17. Setia, M.S. Methodology Series Module 3: Cross-Sectional Studies. *Indian J. Dermatol.* **2016**, *61*, 261–264. [CrossRef] [PubMed]
18. VIII-500 Lietuvos Respublikos Gyvūnų Gerovės ir Apsaugos Įstatymas. (Žin. 2012, Nr. 122-6126). Available online: https://e-seimas.lrs.lt/portal/legalAct/lt/TAD/TAIS.46424/asr (accessed on 12 November 2020).
19. 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]

20. 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]
21. 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]
22. Šumilo, D.; Bormane, A.; Asokliene, L.; Vasilenko, V.; Golovljova, I.; Avsic-Zupanc, T.; Hubalek, Z.; Randolph, S.E. Socio-Economic factors in the differential upsurge of tick-borne encephalitis in central and Eastern Europe. *Rev. Med. Virol.* **2008**, *18*, 81–95. [CrossRef]
23. Šumilo, D.; Bormane, A.; Asokliene, L.; Lucenko, I.; Vasilenko, V.; Randolph, S. Tick-Borne encephalitis in the Baltic States: Identifying risk factors in space and time. *Int. J. Med. Microbiol.* **2006**, *296*, 76–79. [CrossRef]
24. Jaenson, T.G.T.; Hjertqvist, M.; Bergström, T.; Lundkvist, A. Why is tick-borne encephalitis increasing? A review of the key factors causing the increasing incidence of human TBE in Sweden. *Parasites Vectors* **2012**, *5*, 184. [CrossRef]
25. 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. *Vet. Microbiol.* **2009**, *135*, 267–271. [CrossRef] [PubMed]
26. Stefanoff, P.; Pfeffer, M.; Hellenbrand, W.; Rogalska, J.; Rühe, F.; Makówka, A.; Michalik, J.; Wodecka, B.; Kiewra, D.; Dobler, G.; et al. Virus Detection in Questing Ticks is not a Sensitive Indicator for Risk Assessment of Tick-Borne Encephalitis in Humans. *Zoonoses Public Health* **2013**, *60*, 215–226. [CrossRef] [PubMed]
27. Gäumann, R.; Mühlemann, K.; Strasser, M.; Beuret, C.M. High-Throughput procedure for tick surveys of tick-borne encephalitis virus and its application in a national surveillance study in Switzerland. *Appl. Environ. Microbiol.* **2010**, *76*, 4241–4249. [CrossRef] [PubMed]
28. 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]
29. 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]
30. Balling, A.; Plessow, U.; Beer, M.; Pfeffer, M. Prevalence of antibodies against tick-borne encephalitis virus in wild game from Saxony, Germany. *Ticks Tick Borne Dis.* **2014**, *5*, 805–809. [CrossRef]
31. 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*, 78. [CrossRef]
32. Klaus, C.; Beer, M.; Saier, R.; Schubert, H.; Bischoff, S.; Suss, J. Evaluation of serological tests for detecting tick-borne encephalitis virus (TBEV) antibodies in animals. *Berl. Munch. Tierarztl. Wochenschr.* **2011**, *124*, 443–449.
33. Pacilly, F.C.A.; Benning, M.E.; Jacobs, F.; Leidekker, J.; Sprong, H.; Van Wieren, S.E.; Takken, W. Blood feeding on large grazers affects the transmission of *Borrelia burgdorferi* sensu lato by *Ixodes ricinus*. *Ticks Tick Borne Dis.* **2014**, *5*, 810–817. [CrossRef]
34. Rizzoli, A.; Hauffe, H.C.; Tagliapietra, V.; Neteler, M.; Rosà, R. Forest Structure and Roe Deer Abundance Predict Tick-Borne Encephalitis Risk in Italy. *PLoS ONE* **2009**, *4*, e4336. [CrossRef]
35. 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. *Int. J. Parasitol.* **2012**, *42*, 373–381. [CrossRef] [PubMed]
36. Hofmeester, T.R.; Sprong, H.; Jansen, P.A.; Prins, H.H.T.; van Wieren, S.E. Deer presence rather than abundance determines the population density of the sheep tick, Ixodes ricinus, in Dutch forests. *Parasites Vectors* **2017**, *10*, 433. [CrossRef] [PubMed]
37. Gassner, F.; Verbaarschot, P.; Smallegange, R.C.; Spitzen, J.; Van Wieren, S.E.; Takken, W. Variations in Ixodes ricinus density and Borrelia infections associated with cattle introduced into a woodland in The Netherlands. *Appl. Environ. Microbiol.* **2008**, *74*, 7138–7144. [CrossRef] [PubMed]

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