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**A 4-year study of a natural tick-borne encephalitis virus
focus in Hungary and the joint epidemiological
analyses, and laboratory surveys**

Doctoral Theses

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1. Introduction

Tick-borne encephalitis (TBE) is a viral arthropod-borne disease, and one of the most important zoonoses in Eurasia, with 157,584 TBE cases documented in Europe and Russia; between 1990 and 2007. This represents an average of 8,755 cases per year in Europe across the 18-year period, with increased incidence over the last decade in most European countries. However, the overall number of TBE cases may be underestimated, because case-reporting systems differ among countries and are highly inconsistent. TBE virus (TBEV) belongs to the family *Flaviviridae* and causes disease in a wide geographical range from the eastern boundary of France to Japanese islands.

TBEV is maintained in nature in a complex cycle involving the virus itself, ticks (*Ixodes ricinus* in Hungary) as vectors and small rodents as hosts of ticks. The survival of the virus can only be successful in natural focuses where these factors are present. The virus replicates and overwinters in ticks, and is maintained through both transovarial and transstadial transmission. In Hungary, the main reservoir of the virus is yellow-necked field mouse (*Apodemus flavicollis*) and bank vole (*Myodes glareolus*). Uninfected ticks can acquire TBEV during brief periods of viremia in rodent hosts, and by co-feeding with infected ticks on the same host. Humans are infected through tick bites or following the consumption of raw milk of infected ruminant species (mostly goats).

In humans, the clinical symptoms are fever, headache and weakness, while the course of the disease (even in cases with inflammation in the nervous system) is usually benign. Infection is predominantly asymptomatic, though 10-25% of cases are hospitalized with neurological signs, and 1-2% of the hospitalized cases suffer long-term paralysis or death.

In Hungary, TBE is reported to the National Centre for Epidemiology, and recorded in the National Database of Epidemiological Surveillance System using the infectious disease report card completed by the physician who established the medical diagnosis. According to this database, the number of confirmed cases decreased: the previous annual average of 262 dropped suddenly to 68 during the period from 1997 to 2008.

In recent years, TBE was intensively researched in endemic areas across Europe.

Between 1952 and 1981, Erzsébet Molnár coordinated the surveys of TBE natural focuses in Hungary. As a result, 60 different virus strains were isolated (from questing nymphs, small mammals, and two deceased persons as well). Rate of the ticks carrying the virus was 0.5‰. Since that, this work was not continued in Hungary. We have neither new virus isolation, nor new data on the virus prevalence in ticks.

Based on that, a more detailed examination of TBE epidemiology in Hungary is needed. Our study site (a natural TBEV focus) was determined just on a former goat pasture, where from a goat milk-borne TBE outbreak started. During a 4-year study of this natural focus, our aims were as follows:

- a) Collection of ticks and rodents living at the study site, on a regular basis, monthly between April and October. To note essential meteorological data (temperature, precipitation).
- b) Identification of the stage and species of collected ticks. Live-trapping of rodents and taking blood by the retroorbital bleeding method and releasing the animals after marking. Determination of the species composition and their seasonality.
- c) Processing the sampled ticks in laboratory for isolation experiment. Determination of seropositivity of rodents.
- d) Isolation of the TBEV.
- f) Description and characterization of the natural foci by using these data.

In order to gain more information on knowledge relating to TBE epidemiology and transmission, the following objectives were also set:

- g) Epidemiological analysis of the available epidemiological data collected routinely, in particular the examination of spatial component of the disease distribution. Studying the previously identified alimentary transmitted TBE cases.
- h) To examine the diel questing activity of *Ixodes ricinus*.
- i) To study the virus infection induced in a free-ranging wild rodent species by histological and serological methods, in order to better understanding their role played as reservoir.

2. MATERIALS AND METHODS

1. Analysis of data recorded in the national infectious disease reporting system

Morbidity and corresponding demographic data (sex, age, place and date of disease onset) between 1998 and 2008, were obtained from two sources, the National Centre for Epidemiology and National Database of Epidemiological Surveillance System. Population data at the municipality level, by sex and age groups from 1998 to 2008, were obtained from the National Institute of Environmental Health. Age in the population data set was recorded in intervals: One group for each year between ages 0–4, then 1 group for each consecutive 5 years from age 5 to 85, and finally 1 age group for age 85 and above. To analyse **trends** in incidence the direct standardized incidence rates were used per 100 000 person years. The reported annual morbidity data were tabulated in weekly periods (1–52) between 1998 and 2008. The **spatial patterns** of TBE and Lyme disease (LD) incidence were investigated at the municipality level. Indirect standardized incidence ratios (SIR) smoothed using hierarchical Bayesian methods were calculated by taking the age-specific incidence rates of the Hungarian population as a reference; risks were adjusted for age. Homogeneity and linear trend tests were carried out to check whether the risk is statistically homogeneous across bands and to test the global association of the latitude/distance and the relative risk of incidence due to TBE and LD).

All known **milk-borne TBE** epidemics (and which had a history with possible raw milk and/or milk products consumption, revealed by the epidemiological investigation) or individual infections confirmed by laboratory methods between 1953 and 2011 were involved in our study.

Microsoft® Office Excel 2003 was used for systematizing the data. The spatial epidemiological studies were performed using Rapid Inquiry Facility (RIF 3.1.2), a free extension of ArcGIS Desktop. The topographical modeling was performed using ArcGIS version 9.3.

2. A 4-year study of a TBEV focus

The study was carried out next to Lakhegy, Zala county, Hungary. The **sampling site** was located in a former plum plantation abandoned 20-25 years ago. By a preliminary serological study a relatively small (0.49 ha) site with seropositive mice was established as future study area around a hunting feeding point. Next year, the sampling area with forty-nine 10x10 m² study plots was marked off for regular sampling of questing ticks and small rodents, in seven lines along the former plum tree rows. The study area was visited 7 months a year from April to October between 2010 and 2013, and also in March and November when the weather permitted. A second study was established (without preliminary serological survey) of the same size and study plot system was established 177 m away from the sampling site, still in the plum orchard. This second area contained newly established (2010) feeding point for deers and boars in the middle.

Recording the **climatic factors** of the area, minimum, maximum daily temperature, and precipitation data were gathered from the nearest (Zalaegerszeg) meteorological station on daily basis. In addition to that, in 2012, two Tinytag Plus-2 devices were placed at our sampling site on the ground, for measuring air temperature and humidity at every 15 min throughout the year.

Ticks were sampled by the cloth-dragging method, with a 1 m² white flanel cloth. Five, 2-m-long drags were performed per study plots, and ticks were carefully collected by tweezers into a microcentrifuge tube, marked with the number of the plot. Tubes were stored in a cooler box until the end of the day, and then transported to the laboratory, where ticks were identified according to species and developmental stages by light microscope. Tick samples were pooled according to study plots, in order to keep together ticks from the most neighboring territories in one pool. Individuals of different tick species and developmental stages were never combined in one pool. Smashed in sterile porcelaine mortars, ticks were suspended in 200 µl (larvae and nymphs) or 400 µl (adults) steril phosphate buffered saline. Twenty microliters of this solution was inoculated into 5–7 days old suckling NMRI mice. For 3 weeks the mice were examined daily for clinical signs.

Small mammals were trapped using wooden rodent live traps. At each visit, the live traps were set in the middle of each study plot, and left there for two days and two nights. Traps were checked twice a day: in the morning and in the afternoon. At first capture, each animal was assigned a unique identification number and marked with a unique toe-clip pattern. 200–400 µl of total blood was taken by the retroorbital bleeding method from adult rodents over 17 g bodyweight. Finally, animals were released at the point of capture and traps were set again. After 10 h of incubation at 4 °C, the blood samples were centrifugated at 11,000 rpm for 1 min, the supernatant sera were separated and frozen at -70 °C prior testing. Sera were transported to our laboratory in cooler box, where they were kept frozen until further assays. Rodent sera were tested for TBEV-specific antibodies by virus neutralization. Ten microliters of undiluted serum was mixed and incubated with 50 plaque forming units of TBEV (propagated and titrated on N2a mouse neuroblastoma cell line) in duplicate wells of flat bottomed 96-well tissue culture plates for 1.5 h at 37 °C in CO₂ thermostate. Then N2a cells were added in 200 µl culture medium to all wells and plates were incubated for a week. Virus control and cell control wells (2–2) were incorporated in every plates. After the incubation period, the wells were checked for cytopathic effect.

3. Monitoring the diel activity of ticks

The sampling site was located in the above described former plum plantation. Between the former tree rows, twenty-four 5x5 m study plots were marked out for sampling. One day of every month from April to October (between 5th and 15th days of the month) in 2012, ticks were sampled throughout the day hourly, by the cloth-dragging method (with a 1 m² white flanel cloth) from the whole 25 m² area of the study plots, from one plot at every hour. Ten drags were performed per study plots and ticks were carefully collected by tweezers into a microcentrifuge tube after each drag. They were stored and identified as it is detailed under point 2. Twenty-one small mammal traps were set in three rows: 7 on the western edge of the site, 7 in a central row, and 7 more along

the eastern edge. At times of sampling, hourly, all rodent traps were checked. Captured animals were carefully set out into a little textile bag in which they were took by scruff and held such way until the species and sex was examined and registered. Then animals were unhande at the point of capture and traps were set again. Local air humidity and temperature were recorded by TESTO 605 H1 device at ground level at the edge of the sampled plot hourly, in line with tick samplings. Daily sunrise and sunset values of Szombathely (same longitude as the study site) were available on the official homepage of the Hungarian Institute for Meteorology.

4. Study of the dosiss dependence of TBEV infection in susceptible and resistant murine species

Individuals of two animal species *Apodemus agrarius* and NMRI laboratory mice were challenged perorally and intramuscularly with wide range of infective dosis. A total of nine infective doses were applied: 10^{-4} , 10^{-3} , 10^{-2} , 10^{-1} , 1, 10, 10^2 , 10^3 and 1500 plaque-forming units. To study the viral pathogenesis before and after initiation of serological immune response, two individuals of both species were infected with a certain dosis, were sacrificed (in CO₂-chamber at post infection day 7. Before sacrificing blood samples were taken for serological studies. Clinical signs were recorded daily. The infective virus was one of the isolations from Lakhegy, from *I. ricinus* larvae (2011) propagated and titrated on N2a mouse neuroblastoma cell line.

For **immunohistochemical staining**, serial tissue sections were deparaffinised, treated in citrate buffer (pH = 6.0) in a microwave oven at 750 W for 20 min, incubated at room temperature in 3% H₂O₂ solution for 10 min, and then with 2% solution of milk powder for 20 min. The sections were incubated at 4 °C overnight with TBEV-specific mouse antibody kindly provided by Matthias Niedrieg. The colour reaction was developed by ARK Animal Research kit HRP. The sections were counterstained with Mayer's haematoxylin, and examined at 200x magnification.

Virus neutralization was performed using 100 plaque-forming units virus, as it was described in point 2. After the incubation period (1 week), the wells were checked for cytopathic effect. In case of doubtful cytopathic effect the presence or absence of live virus were proved by an animal experiment with inoculation.

3. RESULTS

1. Analysis of data recorded in the national infectious disease reporting system

Overall, 703 TBE cases were recorded in the infectious disease reporting system in the 11 years between 1998 and 2008 in Hungary (annual average of 64 TBE cases). The incidence of TBE was characterized by a W-shaped fluctuation with relative minima in 2000 and 2005. The TBE cases occurred between weeks 17 and 34 (May–August), with a culmination between weeks 23 and 26 (June). A second explicit peak in the number of cases (in autumn) could be observed in case of TBE. More than two-thirds (68%, 478 cases) of the reported TBE cases were males; only 32% (225 cases) were females. On the other hand, the LD cases had a nearly equal sex ratio (53%). The age-specific TBE **incidence rate** was characterized by a predominance of men in all age groups during the study period. The rates were elevated in boys aged 2–4 and in men aged 35–39 and 45–49 years. The age-specific morbidity rates due to LD were considerably different: the highest morbidity rates due to LD were determined for females ages 45–64 and for children ages 2–9. The **spatial distribution** of the smoothed SIR values was characterized by a strong spatial structure. The areas of significant excess morbidity risk were located in the southwestern part of Hungary and in areas near the central section of the northern border of the country for TBE. The TBE incidence rate assigned to localities above 200 meters was 10.93 [95% CI 9.37–12.75] times higher than those below 114 meters. The incidence in municipalities falling between 140 and 155 meters of altitude was equal or already higher than the national average incidence (LD, 1.031 [95% CI 0.994–1.069], TBE, 1.404 [95% CI 1.224–1.610]). The results of the risk analysis showed a strong association between the risk of morbidity due to both diseases and their distance from high forests (forests exceeding 150 meters of altitude) (TBE: $\chi^2_{\text{Homogeneity}} = 862.29$, $p = 0$, $\chi^2_{\text{Linearity}} = 841.59$, $p = 0$; LD: $\chi^2_{\text{Homogeneity}} = 1463.49$, $p = 0$, $\chi^2_{\text{Linearity}} = 1427.89$, $p = 0$) risks showed a decreasing tendency with increasing distance from high altitude forests for both diseases.

Between 1953 and 2011 15 years were found in which **food-borne TBE** infections occurred in Hungary. Overall 27 independent events of infection were identified, 9 (33%) sporadic or individual, 15 (56%) familiar and 3 (11%) larger epidemics affected more than 5 persons. The total number of cases was 111. The seasonal and spatial distribution of cases and the predominance (55,1%) of men were similar to the one occurred among the cases by direct tick bite. Spatial distribution of goat milk-borne cases and events of infection did not coincide with the distribution of goats reported in the country.

2. A 4-year study of a TBEV focus

In **area 1** a total of 7,247 ticks were sampled and three tick species (*Ixodes ricinus*, *Haemaphysalis concinna* and *Dermacentor reticulatus*) were identified. Overall 84.6% of the collected ticks were *I. ricinus*, mostly in subadult forms: 38.3% larvae, 54.0% nymphs 7.7% adults. The seasonality of

these tick species varied throughout the studied period, but in 2011 and 2012 an apparent nymphal peak could be observed in spring (between April-June) and in late summer (between September-October), while larvae showed questing activity mainly in summer with highest peak in August. In 2010, seasonality data were distorted by the weather. Overall 541 small rodents were trapped and four rodent species (*A. flavicollis*, *A. agrarius*, *M. glareolus* and *Microtus subterraneus*) were identified, with densities of 78-165 individuals/year/0.5 hectare. Over the four years the annual number of captured rodents were almost identical, but the proportion of different species varied by year. Bank voles (*M. glareolus*) were first observed in 2011 (2nd study year), constituted 6% of the sampled population in 2011-2012 and 23% in 2013. Proportion of recaptured rodents marked in the previous year were 1.3-7.3%, which confirms short lifespan of rodents in nature (1-1.5 years as maximum).

TBEV were isolated three times from pools of the 7,247 sampled ticks (in August 2011 from *I. ricinus* larvae; in May 2012 from *I. ricinus* nymphs, and in July 2012 from *H. concinna* nymphs). The study plots from where the viruses were isolated did not coincide, but were about 20 meters from each other.

Twenty-eight of 539 tested sera (5.19%) were seropositive for TBEV. Seropositivity of *M. glareolus* (20.5%) was 5.5 times higher than that of *A. flavicollis* (3.7%) and 4.5 times higher than that of *A. agrarius* (4.6%). The differences between the two mice species and the bank vole were significant ($p = 0.0004$ for *A. flavicollis*, $p = 0.0027$ for *A. agrarius*). Seropositivity of sexes did not differ in *A. agrarius* and *M. glareolus*. In *A. flavicollis* 4.5 times higher positivity was observed in females than in males though this difference was not significant (6.7% vs. 1.5%, $p = 0.0530$). Significant ($p = 0.0117$) difference was found between seropositivity of adult (6.8%, $n = 366$) and subadult (1.7%, $n = 175$) rodents. Study plots where virus was isolated from, and plots with positive rodents did not coincide.

From **area 2**, fewer ticks (2,369 specimens) and rodents (375 individuals) were captured and positivity of rodents was lower (4.93%; 14 from 284 tested sera) compared to area 1, and no virus strain was isolated.

The **differences between the prevalences found in the two areas** were not significant (χ^2 test: $p = 0.7487$). The seasonal fluctuation of the TBEV seropositive rodents and the seasonality of subadult forms of *I. ricinus* was overlapped.

3. Monitoring the diel activity of ticks

1,063 *I. ricinus* (408 larvae, 598 nymphs, 34 females, 23 males) were collected, and rodents were captured 28 times over the 7 months on the sampled territory. 71.4% of rodents (20/28) were trapped in the first 6 h of the day (23:20–5:20 h). From April to October questing activity of nymphs increased in the **3 h after sunrise**, comparing to their activity in the 3 h-period before sunrise. The difference was statistically significant (paired t-test: $p = 0.022$). We compared the questing activity (number of sampled *I. ricinus* nymphs) in the **3 h before sunset and the 3 h after sunset**. Activity

was dominant before sunset in April and May, almost equal in June, then activity moved to the after-sunset hours in July-Aug-September, in October again, balanced activity was shown before and after sunset. Proportion of nymphs sampled 3 h after sunset/total sampled nymphs 3 h before and 3 h after sunset showed statistically significant correlation to activity of rodents. Pearson's correlation coefficient was 0.81 (Pearson's correlation test: $p = 0.026$). Daytime activity of *I. ricinus* nymphs turns to evening-night activity just in the months, when the nocturnal rodent species are at the top of their population density (July–September). April–July both nymphs and larvae showed higher daytime activity, while this turned to dominant night activity in August–September, while activity changed to equal in day and night in October. The changes were statistically significant, for nymphs and larvae (Pearson's χ^2 test: $p < 0.0001$).

4. Study of the dose dependence of TBEV infection in susceptible and resistant murine species

Clinical signs (from slowness to complete inactivity-tremor) were evident only in NMRI mice in both per os and intramuscularly infected groups. All *A. agrarius* individuals were free of any clinical signs of encephalitis. By **intramuscular infection** in NMRI mouse the viral antigen was detected in few neurons in cerebral cortex and brainstem and in the autonomous ganglia of the small intestine. Histological lesions (stained with haematoxylin and eosin) were not observed in this case. No TBEV antigen or any histological alterations were detected in intramuscularly infected *A. agrarius* individuals. By **per os infection** in NMRI mouse large number of infected cells were detected by immunohistochemical staining in all part of the brain and low to moderate number of infected cells were evident in the autonomous ganglia of the intestine and/or stomach in all of these cases. Histological lesions (focal acute malacia in the midbrain) appeared. The cytoplasm of neurons were frequently vacuolated, their number was reduced and lympho-histiocytic infiltration were observed. No histological lesions were seen in other visceral organs. TBEV antigen was found in the brain of the per os infected striped field mice. Few infected neurons and glial cells in the olfactory bulb and in the anterior olfactory nucleus and the presence of mild lympho-histiocytic vasculitis restricted to this area was evident. Few infected neurons and glial cells were observed in the caudate putamen, which was accompanied by mild lympho-histiocytic vasculitis in the same area. Necrosis of few neurons and presence of glial nodules was also present in the brainstem in this case. No histological lesions were observed in other organs.

5. New scientific results

Results of our 4-year fieldwork and laboratory investigations, could be summarized as follows:

1. Our study was the first joint analysis of the reported TBE and LD cases at the municipality level on comparative basis in Hungary. Using spatial epidemiological methods we found that:
 - the most important risk areas of both diseases were identified;
 - disease risks showed a decreasing tendency with increasing distance from forests exceeding 150 meters of altitude.
2. Our study was the first comprehensive descriptive epidemiological analysis of all known alimentary transmitted TBE cases in Hungary.
3. Our 4 year study of a TBEV natural focus revealed novel data:
 - prevalence of virus in ticks is low (3 positive pools of the 7247 sampled ticks in area 1, 0,414‰);
 - the cumulative seropositivity of small mammals in the two areas was 5,1% (42 of 823 tested sera);
 - at a certain time TBEV hot spots are restricted to some m² territory, and continuously alter as infected unfed ticks die, or where engorged infected ticks fall off from their hosts;
 - measured climatic factors did not seem to play role in virus prevalence;
 - bank voles trapped in the area showed higher seropositivity rates and were parasited by more ticks comparing to the *Apodemus* species, which indicates that bank voles have more important role in distribution and maintenance of the virus than mice;
 - *Haemaphysalis concinna* nymphs also carry the virus in nature.
4. daily pattern of questing activity of *Ixodes ricinus* was examined over 7 months at a targeted fieldwork. The followings were statistically proved:
 - questing activity of *Ixodes ricinus* nymphs grows after sunrise;
 - the seasonal activity of ticks shows significant correlation to increasing activity of rodents during the night.
5. In an animal experiment we found, that
 - individuals of *Apodemus agrarius* inoculated by higher infective virus dosis undergone subclinical encephalitis.

4. DISCUSSION

Analysing the epidemiological situation of TBE and LD cases recorded in the national infectious disease reporting system between 1998 and 2008, we found that the vast majority of the patients of TBE were males, which is congruent with previous data on that, though the main reason for this sex bias phenomenon is unknown. The spatial components of reported and serologically confirmed TBE cases were previously examined in Hungary; however, our study is the first to include LD cases as well. The simultaneous analysis of the higher-altitude locations (at least to a certain elevation) and the forest coverage added a new aspect to the definition of a typical transmission area. The human habitats lying in the high-altitude or in the immediate vicinity (0–1 km) of a high-altitude region can be considered as exposed to significant risk. Previously, it was stated that the forest cover is related to the number of reported TBE cases in each Hungarian county, and the proximity of human habitats and forests explained the observed pattern of TBE incidence in Hungary. Our results confirmed these findings in the case of TBE and demonstrated these for the first time in the case of LD. It is clear that unreported morbidity cases can be a major limitation of studies based on routine surveillance data. Geocoding of cases was based on reported residence of the patient instead of probable location of infection. Because the reported cases denoted strong spatial structure, a short effective distance between these two places was assumed. The methods used and mentioned within this study may help to identify more accurately, based on specific age and gender groups, the **population at high risk**.



In 1992-2011 a total of 2280 cases of TBE were reported from which 100 (4,4%) were **alimentary transmitted** by raw goat or cow milk. This proportion fits the similar rates observed in other Central European countries. We found, that decreasing tendency occurred in cases acquired by direct tick bite, can not be observed in alimentary transmitted cases. 58% of total registered food-borne infections was observed between 1997 and 2011. Year 2007 was exceptional, as half of the total confirmed cases belonged in the epidemic started from Lakhegy, Zala county. Proportion of milk-borne infections from the total number of cases was considerably increased during the period analysed. All of the 27 alimentary outbreaks occurred in the north-central and south-western parts of Hungary, the same areas we identified as natural focal points by spatial analysis. Unfortunately, the epidemiological identification of human infections only rarely associated with in-depth analysis of the dairy animals linked to the transmission. It is important to emphasize that animals found recently infected by a serological test, should not be excluded from the production of milk for a long time, because these animals will be protected for life starting from their next lactation.



Our results showed that the prevalence of virus in ticks is low (3 isolates from 7,247 ticks, 0.404‰ in area 1). This prevalence is comparable to previous findings of studies from randomly selected parts of Hungary conducted between 1952 and 1978 (from 48,623 unfed *I. ricinus* 23 TBEV strains were found, 0.473‰ prevalence). As we found only 3 positive pools, it is likely that in these pools were only 1-1 positive nymphs and in case of larvae, a minimum of 1 to a maximum of 4-5 individuals carried the virus. This positive pool contained a total of 151 larvae collected in sampling unit 36 in area 1. Three pools were formed from this 151 larvae, containing 50, 50 and 51 larvae respectively. It is probable that the 151 larvae hatched from eggs laid by a single female tick, and acquired their infection by transovarial infection. Most likely it can be assumed that the 3 positive pools meant 3 positive individuals. The low TBEV prevalence among ticks in our sampling area is consistent with data obtained during the veterinary investigation of the milk-borne TBE outbreak linked to the neighbouring goat farm: of the 75 milking goats, active infection could be proven only in one animal by presence of virus specific IgM, after 50 days of grazing on our sampling site.

The trapping location of infected animals were nearby, mostly restricted to the study plots with positive ticks. The two seropositivity peaks in rodents observed in May-June and September-October just followed the activity peak of nymphs in spring and of larvae in August. The low overwintering success (1-7%) and seroprevalence (5%) in rodents suggest that probably only seronegative individuals start to build up the population in each spring, so a seropositive rodent should be considered as being infected in the year of capture. Coincidence of seropositive rodents and number of sampled ticks (in April and August) proves the key role ticks play in transmitting the virus to rodents, and also that detectable level of antibodies is produced short after the infection small mammals. **At a certain time the area at risk (TBEV „hot spots”)** is restricted to some m² territory, where an infected nymph or larvae hatched from an infected egg batch are just questing. These hot spots continuously alter as infected unfed ticks die, or where engorged infected ticks fall off from their hosts. As a consequence, it is not possible to predict which parts of the focus are or will be dangerous for humans at a certain time. While it is can not be said with certainty where a positive tick or positive rodent is at a given time, by knowing where areas of high tick survivability and host preference co-exist, you can predict types of habitat where humans could be at risk at the times of year when human cases peak. Climate (except dew) did not seem to play role in virus prevalence.



Questing activity of *I. ricinus* ticks grows just **after sunrise** throughout the year (except September, but low number of sampled ticks and cloudy sky could be reason for that). Increased activity of rodents at and after sunrise is shown (5 animals were captured at the 3 h period after

sunrise throughout the year from the 28 captures). Without direct data we suspect that light at every dawn could cause this increased questing activity. As proportion of nymphs sampled for 3 h after sunset/total sampled nymphs for 3–3 h before and **after sunset** showed statistically significant correlation with activity of rodents, we suspect, that ticks somehow feels the dynamics of the local rodent populations. As we experienced rodents of the sampling area almost inactive in daytime, activated at sunset and at night. Daytime activity of nymphs turns to evening-night activity just in the months, when the nocturnal rodent species are at the top of their population density (July–September).



Our study first showed that **large amount of infective virus** reaches and replicate in cells of the central nervous system in a free-ranging wild murine *Apodemus* species without clinical signs. This result show, that under certain circumstances murine species are also sensitive for subclinical encephalitis, not only voles. This (and the recovered subclinical pattern of the infection) proves, that immune functions stand behind the various outcome of TBEV infection in murine species. In *A. agrarius* the immune system is able to prevent the central nervous system from viral invasion of relatively small amount of virus and also able to clear virus from the the central nervous system without clinical illness. The lack of serological response in *A. agrarius* infected by low infective doses indicates that their effective local immune response overcomes the invading viruses at the inoculation site, the skin. Continuous lack os serological response in NMRI mice infected by several infective doses reveales their weak local immune response. Fast invasion of their central nervous system does not leave enough time for effective serological response, or the produced IgG-s at the periphery can not help the infected individual to overcome TBEV encephalitis.

5. PUBLICATIONS RELATED TO THE TOPIC OF THE PRESENT THESIS

Full text papers in peer-reviewed journals

- Zöldi Viktor, Juhász Attila, Nagy Csilla, Papp Zoltán, Egyed László: **Tick-borne encephalitis and Lyme disease in Hungary: the epidemiological situation between 1998 and 2008**, *Vector-Borne and Zoonotic Diseases*, (13)4: 256-265, 2013. doi:10.1089/vbz.2011.0905., *IF₂₀₁₃*: 2,531
- Zöldi Viktor, Ferenczi Emőke, Egyed László: **Tej közvetítette kullancsencephalitis-járványok Magyarországon**, *Magyar Állatorvosok Lapja*, 135. 48-56, 2013. *IF₂₀₁₃*: 0,185
- Zöldi Viktor, Reiczigel Jenő, Egyed László: **Monitoring the diel activity of *Ixodes ricinus* ticks in Hungary over three seasons**, *Experimental and Applied Acarology*, 61(4): 509-517, 2013. doi:10.1007/s10493-013-9708-4, *IF₂₀₁₃*: 1,821
- Zöldi Viktor, Papp Tibor, Rigó Krisztina, Farkas János, Egyed László: **A 4-year study of a natural tick-borne encephalitis virus focus in Hungary, 2010-2013**, *EcoHealth*, doi:10.1007/s10393-014-0969-0, *IF₂₀₁₃*: 2,267
- Zöldi Viktor, Papp Tibor, Reiczigel Jenő, Egyed László: **Bank voles show high seropositivity rates in a natural TBEV focus in Hungary**, *Infectious Diseases*, doi:10.3109/00365548.2014.975743, *IF₂₀₁₃*: 1,640

Impact factors in total: 8,444

Oral presentations on national conferences

- Zöldi Viktor, Szilágyi Andrásné: **Elegendő-e a bejelentett megbetegedések számának ismerete a kullancsok által terjesztett fertőző betegségek hazai járványügyi helyzetének megítéléséhez?**, *a Magyar Zoonózis Rudnai – Kemenes Tudományos Ülése*, Ráckeve, 7-9 October 2008.
- Zöldi Viktor: **Milyen módon előzhető meg az emberek kullancsossága?**, *a Magyar Parazitológusok Társaságának „Kullancsokkal és az általuk terjesztett kórokozókkal kapcsolatos újabb eredmények” című előadói ülésén*, Budapest, 28 April 2009.
- Zöldi Viktor, Juhász Attila, Nagy Csilla, Szilágyi Andrásné, Páldy Anna: **A Lyme-kór és a kullancsencephalitis incidenciaterületi egyenlőtlenségeinek térinformatikai vizsgálata a fertőzőbeteg-jelentő rendszer (EFRIR) adatai alapján, 1998-2008.**, *a Magyar Zoonózis Társaság által szervezett Szent-Iványi – Binder Napok és Rudnai – Kemenes Tudományos Ülés*, Tiszafüred, 14-16 October 2009.
- Zöldi Viktor, Juhász Attila, Nagy Csilla, Szilágyi Andrásné, Páldy Anna: **Tick-borne encephalitis and Lyme borreliosis in Hungary – The epidemiological situation between 1998 and 2008**, *European Scientific Conference on Applied Infectious Disease Epidemiology (ESCAIDE)*, Lisszabon, Portugália, 11-13 November 2010.
- Zöldi Viktor, Nagy Csilla, Juhász Attila, Papp Zoltán: **A kullancsencephalitis és Lyme-kór incidenciája valamint a feltételezhetően magas átviteli kockázatot jelentő területek térbeli eloszlásának összefüggés-vizsgálata**, *a Magyar Higiénikusok Társaságának XL. Vándorgyűlése*, Esztergom, 5-7 October 2011.

- Zöldi Viktor, Papp Tibor, Rigó Krisztina, Ádámszki Szabolcs, Egyed László: **Egy természetes kullancsencephalitis-góc kétéves vizsgálata**, a Magyar Tudományos Akadémia Állatorvos-tudományi Bizottságának és a Szent István Egyetem Állatorvos-tudományi Doktori Iskolájának 38. „Akadémiai beszámoló” ülése, Budapest, 17 January 2012.
- Jelenik Zsuzsanna, Ferenczi Emőke, Melles Márta, Zöldi Viktor: **Tick-borne encephalitis (TBE) – Epidemiology**, *International Scientific Working Group on Tick-Borne Encephalitis (ISW-TBE)*, 14th Meeting, Bécs, Ausztia, 2-3 February 2012.
- Zöldi Viktor, Papp Tibor, Rigó Krisztina, Ádámszki Szabolcs, Egyed László: **Egy Zala megyei természetes kullancsencephalitis-góc vizsgálata**, a Magyar Higiénikusok Társasága által szervezett, VIII. Fialat Higiénikusok Fóruma, Gödöllő, 10-11 May 2012.
- Zöldi Viktor, Juhász Attila, Nagy Csilla: **A kullancsencephalitis és Lyme-kór miatti megbetegedés területi eloszlásának változása Magyarországon, 2000-2011.**, a Magyar Higiénikusok Társaságának XLI. Vándorgyűlése, Esztergom, 3-5 October 2012.
- Egyed László, Zöldi Viktor, Szeredi Levente: **Kullancsencephalitis vírus-fertőzés szövettani vizsgálata vadon élő rágcsáló fajokban és laboregérben**, a Magyar Tudományos Akadémia Állatorvos-tudományi Bizottságának és a Szent István Egyetem Állatorvos-tudományi Doktori Iskolájának 39. „Akadémiai beszámoló” ülése, Budapest, 28-30 January 2013.
- Zöldi Viktor, Egyed László: **Kullancsok 24 órás napi aktivitásának vizsgálata**, a Magyar Tudományos Akadémia Állatorvos-tudományi Bizottságának és a Szent István Egyetem Állatorvos-tudományi Doktori Iskolájának 39. „Akadémiai beszámoló” ülése, Budapest, 28-30 January 2013.
- Zöldi Viktor, Nagy Csilla, Juhász Attila, Papp Zoltán, Egyed László: **Tick-borne encephalitis and Lyme disease in Hungary – the epidemiological situation between 1998 and 2008**, 47th Days of Preventive Medicine – International Congress organised by Faculty of Medicine in Niš, Public Health Institute of Niš, Serbian Medical Society of Niš, Niš, Szerbia, 24-27 September 2013.
- Zöldi Viktor, Papp Tibor, Rigó Krisztina, Farkas János, Egyed László: **Egy természetes kullancsencephalitis-góc 4 éves vizsgálata**, 2010-2013, a Magyar Tudományos Akadémia Állatorvos-tudományi Bizottságának és a Szent István Egyetem Állatorvos-tudományi Doktori Iskolájának 40. „Akadémiai beszámoló” ülése, Budapest, 27-29 January 2014.

Poster presentations on international conferences

- Zöldi Viktor, Juhász Attila, Nagy Csilla, Szilágyi Andrásné, Páldy Anna: **Tick-borne encephalitis and Lyme borreliosis in Hungary – The epidemiological situation between 1998 and 2008**, *European Scientific Conference on Applied Infectious Disease Epidemiology (ESCAIDE)*, Lisszabon, Portugália, 11-13 November 2010.
- Zöldi Viktor, Ferenczi Emőke, Egyed László: **Milk transmitted tick-borne encephalitis epidemics in Hungary**, European Regional Conference on Goats in Hungary and Romania, 2014, Debrecen-Nagyvárad, 7-13 April 2014.