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PREVALENCE AND DETECTION OF FLAVIVIRUSES OCCURRING IN SLOVAKIA

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ABSTRACT

The tick-borne encephalitis virus (TBEV) and West Nile virus (WNV) are arboviruses of the genus *Flavivirus* in the family *Flaviviridae*. Their hosts are vertebratesof which rodents are the reservoirs of TBEV and birds are the reservoirs of WNV. Both viruses are transmitted from reservoirs to mammals by vectors. TBEV is transmitted by ticks (mostly *Ixodes* spp.) and WNV by mosquitoes (mostly *Culex* spp.). Both viruses are capable of infecting mammals, including man. TBEV and WNV are neurotropic, however infection is, in most cases, subclinical or accompanied by only moderate general signs. However, in some cases they can cause serious disturbances of the CNS. Our study focused on the detection of the genomes of TBEV and WNV in vectors by means of the reverse-transcription polymerase chain reaction (RT-PCR). The flavivirus genome was detected by means of oligonucleotides delineating the sequence in NS5 gene that encodes viral RNA-dependent RNA-polymerase. For the detection of TBEV, we used the oligonucleotide pair detecting the structural envelope protein. The positive samples were subjected to the sequence and phylogenetic analysis. The WNV was not detected in any of the pooled samples prepared from 616 mosquitoes captured in the vicinity of the village Drienovec, district Košice-surroundings. The investigation of 676 ticks demonstrated the presence of one strain of TBEV. One blood-fed *I. ricinus* female was obtained from a goat grazing in a pasture in the Dúbrava area close to Prešov. The genetic analysis revealed the presence of a strain close to the endemic strainsof TBEV Hypr and Neudörfl. The results of our study can become a motivation for additional studies in model locations oriented on ecology and circulation of these important zoonotic flaviviruses.

Key words: arbovirus;tick-borne encephalitis virus TBEV; West Nile virus WNV

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INTRODUCTION

The family *Flaviviridae* includes pathogenic viruses of humans and other animals. It comprises the genera *Flavivirus*, *Pestivirus*, *Hepacivirus* and *Pegivirus*. Many representatives of the genus *Flavivirus* are arboviruses transmitted by vectors — ticks or mosquitoes [8]. The WNV was first described in Uganda in 1937 in a woman with signs of CNS disorders and later it was also demonstrated in mosquitoes, birds and horses. Although it was originally described in the tropics, it was later detected in other regions where it became endemic. The first information about the occurrence in Slovakia dates back to the 1960's, when antibodies to the virus were found in human serum. In the period of 1960—1978, WNV was isolated from *Aedescantans* mosquitoes and neutralization antibodies were detected in 5.4 % of the birds, 5.3 % of the small mammals, 1 % of the hares and 4.4 % of free living ungulates [5]. During the years 2008 and 2011,neutralization antibodies were detected in 8.3 % of non-vaccinated horses [7]. Currently, we lack sufficient information on the prevalence and transmission of WNV in Slovakia. The presence of WNV was confirmed in oral and cloacal swabs and in samples from the brain of birds [3]. Although no case of West Nile fever has been recorded in the Slovak territory, the latest research confirmed the transmission of the WNV among birds and indicated the risk of potential transfer of infection to horses and humans [3].

The tick-borne encephalitis virus (TBEV) is one of the most important tick transmitted arboviruses in Europe. In addition to direct tick transmission, it can spread also by the consumption of non-pasteurised milk products. The first mention of TBEV in Slovakia dates back to 1951, when an extensive outbreak occurred in the district of Rožňava associated with the consumption of raw goat milk [12]. Since then,the cases of tick-borne encephalitis have been reported every year. While in 2013 there were reported 163 cases of tick-borne encephalitis in humans, in 2014 the number of cases decreased to 117 and in 2015 to 88 (a decrease of 28 % in comparison to the 5-year average).

Tick-borne encephalitis occurs in all age categories, from infants up to old people. The course of the disease differs, from oligosymptomatic up to life threatening encephalomyelitis or encephalomyeloradiculitis[11].

Currently, flaviviruses have become a relevant topic, not only due to progressing global warming and climate changes, but also because of the new emerging diseases caused by these agents. Because of the increasing importance of diseases caused by flaviviruses in Slovakia, we devoted our study to the occurrence of these viruses in their vertebrate vectors.

MATERIALS AND METHODS

Ticks and mosquitoes

For the determination of the prevalence of TBEV, we used 676 adults, larvae and nymphs of various species of ticks (Table 1) collected in the Slovak districts of: Žilina, Prešov, Bardejov, Košice-surroundings, Ružomberok, Stará Ľubovňa and Námestovo. They were collected by flagging, or directly from animals, including people.

Table 1. Division of the examined ticks according to species and gender

Species	Gender and developmental stage	Total number
Ixodes ricinus	F	202
	M	105
	N	272
	L	80
Dermacentor marginatus	F	5
	M	7
D. reticulatus	F	3
	M	1
Haemaphysalis	F	1
Total		**676**

F — adult female; M — adult male; N — nymph; L — larva

For the examination of the prevalence of WNV,we obtained 616 mosquitoes (Table 2) from Drienovec within the district of Košice. They were captured in May and July 2016 by means of CDC light traps developed by Centres for Disease Control (CDC) using dry ice as an attractant. Individual pools comprised no more than 25 mosquitoes of the same species.

Table 2. Division of the investigated mosquitoes according to species

Species	Number
Aedes cinereus	57
Ae. rossicus	2
Ae. vexans	166
Anopheles claviger	14
Culex pipiens	70
Culiseta annulata	1
Ochlerotatus cantans/annulipes	75
Oc. caspius	8
Oc. cataphylla	6
Oc. geniculatus	1
Oc. leucomelus	14
Oc. punctor	155
Oc. sticticus	47
Total	**616**

Isolation of nucleic acids from ticks and mosquitoes

The vectors were washed in 400 µl 70 % ethanol for 10 min. Then the ethanol was removed and the vectors were washed twice in water free of nucleases. After mechanical homogenization employing either an apparatus Precellys (Bertin, France) or Tissue Lyser (Qiagen, Germany), the total DNA or RNA was extracted using a commercial QIAamp cador Pathogen Mini Kit (Qiagen, Germany), according to manufacturer's instructions.

Reverse-transcription polymerase chain reaction (RT-PCR)

Complementary DNA was prepared by means of reverse transcriptase (Revert Aid H Minus Reverse Transcriptase, Thermo Scientific, Germany) using random hexamers (Thermo Scientific). For PCR (PCR Dream Taq Green PCR Master Mix, Thermo Scientific) we used primers (Table 3) delineating sequences in TBEV envelope protein and RNA-dependent RNA-polymerase of flaviviruses under the following thermal conditions: initial denaturation 95 °C/1 min; 30 cycles 95 °C/30 sec; 50 °C/30 sec; and 72 °C/40 sec. The final extension took place at 72 °C and lasted 5 min.

Table 3. Oligonucleotides used for detection of TBEV on the basis of RNA-polymerase and envelope protein

Primer	Sequence [5´→3´]	PCR product [bp]
TBEV-Eprot-F	GTTCCTGTGGCRCAYATTG	326
TBEV-Eprot-R	CCTGGRGGYARCTGCATYTCTATG	
PanFlavi-NS5-F	WTRGCMATGACWGAYACHAC	599
cFD2*	GTGTCCCAGCCGGCGGTGTCATCAGC	

Source: Scaramozzino et al. [13]

Sequence analysis

After agarose electrophoresis, DNA fragments of presumed length were excised and used for sequence analysis using software Geneious (Biomatters, New Zealand). The partial sequences were compared with the world database by means of BLAST (Basic Local Alignment Search Tool) analysis. Subsequently,the genetic similarity with the TBEV nucleotide sequence was determined using an algorithm Clusta lW, and a phylogenetic tree was produced.

RESULTS

The WNV was not recorded in any mosquito sample. Of the total number of 675 tick samples, a DNA fragment of length of approximately 350 bp was recorded in several cases, however, subsequent re-amplification confirmed the positivity in only one case (Fig. 1). It concerned a blood-fed female of the species *I. ricinus*, collected from a goat in April 2016 in Prešov-Dúbrava. BLAST analysis confirmed the TBEV. This fragment was named TBEV E prot 389C Dúbrava/PO/2016. The sequence of nucleotides agreed to the highest percentage with European subtypes of TBEV (Table 4). The highest agreement was observed with strains Hypr (99 %) and Neudörfl (98 %) and then with strains K23 and Salem (97 %), which was also reflected in the phylogenetic tree (Fig. 2).

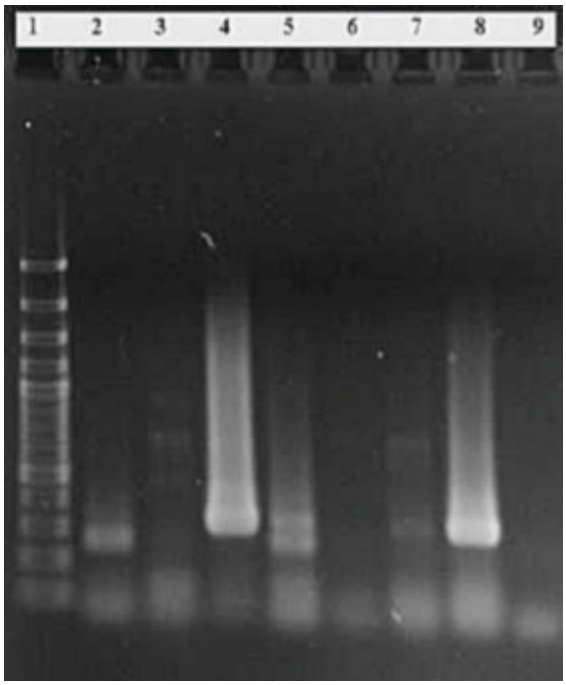

Fig. 1. Result of PCR for identification of TBEV in ticks

Line 1 — 100 bp DNA standard; lines 2—7 — tick samples; line 8 — positive isolation control; line 9 — negative isolation control. Sample in line 4 is unambiguously positive for the presence of TBEV fragment. Samples in lines 2, 3, 5 and 7 were negative after re-amplification

DISCUSSION

Tick-borne encephalitis is a viral zoonotic disease caused by the tick-borne encephalitis virus (TBEV). It is the most important human pathogenicity virus in Eurasia. In the years 1964—1999,the research that was conducted in Slovakia focused on the genus proportion of ticks found in Slovakia. The relevant samples were examined also for the presence of TBEV. This research showed that of 77 000 ticks, 90 % were *I. ricinus*. The TBEV virus was detected only in 0.14 % of the vector [4]. The prevalence of TBEV in ticks in Slovakia has been rarely investigated. The present study examined ticks by the molecular methods. We examined ticks from various regions of Slovakia. No information was available whether the virus was found in these areas in the past. TBEV was detected in one engorged *I. ricinus* female, which is the typical vector. The observed prevalence of TBEV (0.14 %) was similar as that in the study by Grešíková and Nosek [4]. In another study conducted in the Tribeč mountains in the spring of 1964, TBEV was detected in 0.2 % of the 2153 ticks exam-

Table 4. Percentage expression of nucleotide sequence similarity for the partial sequence of envelope protein of the TBEV strains

	TEU39292	Dúbrava	FJ572210	AM600965	TEU27495	AF069066	JN003206	AF527415	JN003205	AB062064	AB062063	NC_005062
TEU39292 TBEV Hypr (EU)		99	99	99	100	85	85	85	87	88	87	86
TBEV Eprot_389C_Dúbrava/PO/2016	99		97	97	98	85	85	84	87	88	87	85
FJ572210 TBEV Salem (EU)	99	97		98	99	85	85	85	86	87	85	86
AM600965 TBEV K23 (EU)	99	97	98		98	85	85	85	86	87	85	86
TEU27495 TBEV Neudoerfl (EU)	100	98	99	98		85	85	84	87	88	87	85
AF069066 TBEV Vasilchenko (SIB)	85	85	85	85	85		100	95	86	86	84	79
JN003206 TBEV Aina (SIB)	85	85	85	85	85	100		95	86	86	84	79
AF527415 TBEV Zausaev (SIB)	85	84	85	85	84	95	95		85	85	85	79
JN003205 TBEV Irkutsk-1861 (FES)	87	87	86	86	87	86	86	85		98	97	81
AB062064 TBEV Sofjin-HO (FES)	88	88	87	87	88	86	86	85	98		97	82
AB062063 TBEV Oshima 5-10 (FES)	87	87	85	85	87	84	84	85	97	97		79
NC_005062 OHFV	86	85	86	86	85	79	79	79	81	82	79	

Similarity of nucleotide sequence of the TBEV strains determined by an algorithm ClustalW. Values in boxes are expressed in per cent: (EU) — European subtype; (SIB) — Siberian subtype; (FES) — Far East subtype; OHFV — Omsk haemorrhagic fever virus

Similarity of nucleotide sequence of the TBEV strains determined by an algorithm Clusta lW. Values in boxes are expressed in per cent: (EU) — European subtype; (SIB) — Siberian subtype; (FES) — Far East subtype; OHFV — Omsk haemorrhagic fever virus.

This phylogenetic tree was produced on the basis of Neighbor-Joining method and the Tamura-Niei model; bootstrap of 1000 replicates; illustrated are only credibility values above 75 %.

ined. In the primary foci in Topoľčianky and Jelenec, the prevalence was 0.5 % and 0.9 %, respectively [4]. In arecent study conducted in Poland, there were examined 471 adult ticks *D. reticulatus* collected in the national park Biebrza, Bialowiezsky forest and Masurian region in North-West Poland. The RT-PCR revealed the prevalence of the virus ranging from 0.99 % to 12.5 % depending on the location and the mean prevalence reached 2.1 % [2]. In another survey investigators examined 87 *I. ricinus* ticks and 148 *D. re-*

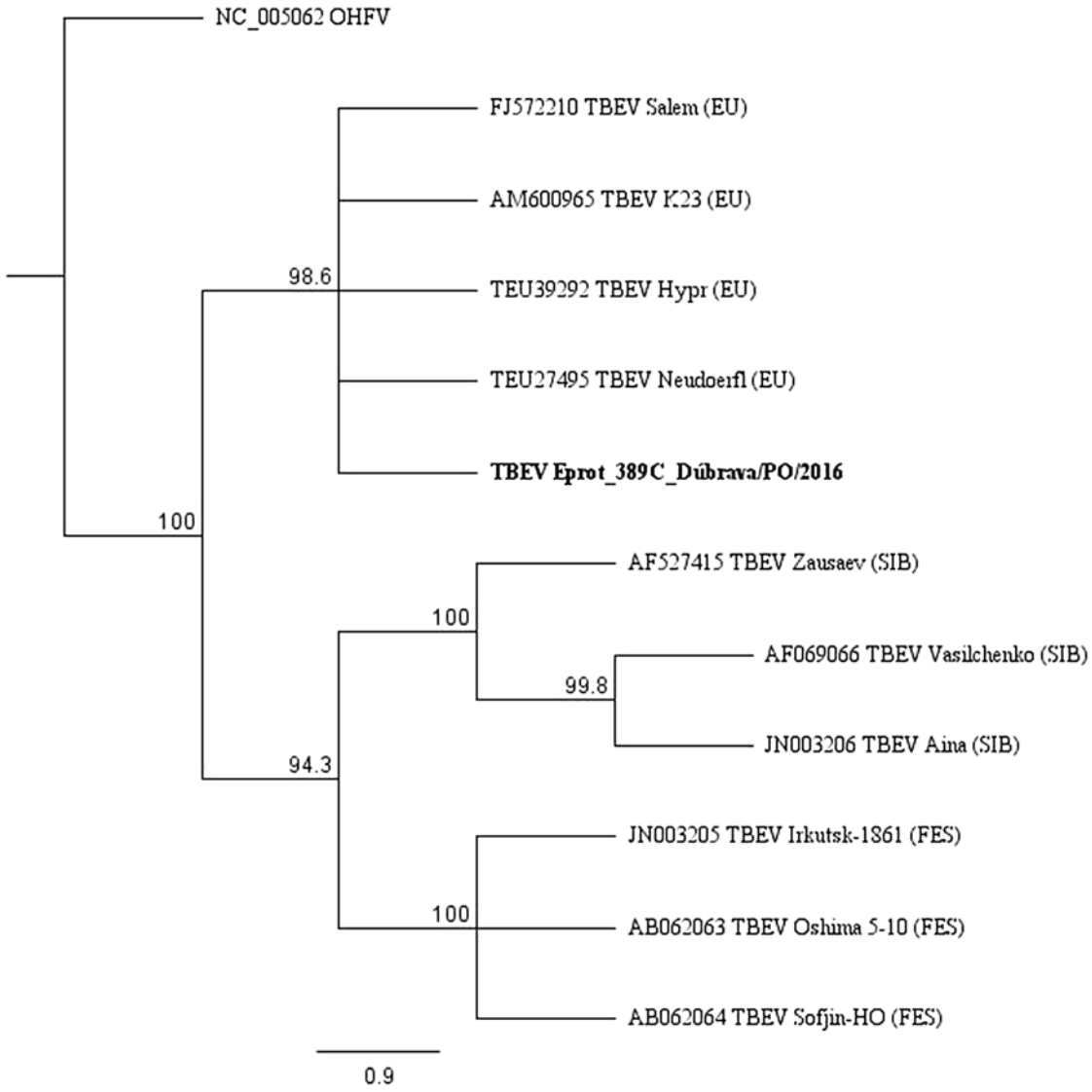

Fig. 2. Phylogenetic tree of the strain TBEV produced on the basis of partial sequence of the envelope protein

ticulatus ticks collected in the period from April to September 2008 and 2009 in the district of Lublin in East Poland. By means of RT- PCR,the virus was detected in 1.6 % of the *I. ricinus* and in 10.8 % of the *D. reticulatus*. The prevalence was lower in males and nymphs than in females. In some locations the virus was not detected[14].

The prevalence of a disease is related to the prevalence of the vector. The spread of *D. reticulatus* can be divided into two areas, western and eastern Europe. Western Europe covers the territory from France up to eastern Germany and eastern Europe spreads from east Poland to Siberia. Since the 1990's, these ticks have been found in areas that were previously free from them[9]. The vector occurred also along the Danube and Morava rivers and in eastern Slovakia, close to Latorica, Bodrog and Tisa rivers. In the recent period, it expanded to the north and occurred along the Laborec river up to the Vihorlat mountains, but also in lower regions of the Váh, Hron and Ipeľ rivers.

I. ricinus occurs throughout Europe, from Ireland to the Urals and from northern Sweden down to northern Africa. In Slovakia, it is spread in the whole territory up to 600—800 m above sea level. Due to global warming, the upper boundary of its occurrence has been shifting gradually to more than 1000 m above sea level [10].

In the recent years, we recorded the continuous spreading of ticks to new areas, particularly into north-east Europe [2]. The positive detection of TBEV RNA in a *I. ricinus* female in Dúbrava in the Prešov district indicates the pres-

ence of a natural focus. In order to confirm its presence, it is necessary to examine also its reservoir, namely small mammals. A positive tick was collected from a grazing goat. Its owner reported the production of cheese from the goat milk. Because information about the occurrence of TBEV in reservoirs in Dúbrava and its potential spreading via alimentary path is absent, additional studies appear desirable.

West Nile fever is a viral zoonotic disease transmitted by mosquitoes. West Nile virus was isolated from 43 mosquito species, particularly of the genus Culex. The principal vectors in Europe are *Cx. pipiens*, *Cx. modestus and Coquillettidia richiardii*. Virus isolates from haematophagous arthropods have been occasionally reported. West Nile fever has been known in Europe for several decades. Cases were reported mostly in the Mediterrean region and south-east Europe, but also in Italy, Romania, France, Hungary and Austria [7, 15].

In 2008—2009 research was carried out in Hungary and Austria on 19 pools from Hungary, each consisting of 2–25 mosquitoes, and 4983 mosquitoes from Austria, of the species *Cx. pipiens*. The investigated mosquitoes were collected in south-east Hungary and from the vicinity of Vienna, upper and lower Austria, Burgenland and Steiermark in September and October of 2008. The WNV genome was detected in 7 pools by the RT-CPR method. All positive mosquitoes were *Cx. pipiens* females collected in lower Austria, close to the location associated with the mortality of birds caused by WNV. Data from the preliminary surveillance of mosquitoes indicated a similar degree of infection in mosquitoes involving lineage 2 (approx. 5 %) in comparison with lineage 1 in the endemic regions. Because this research focused mainly on mosquitoes *Cx. pipiens* and only a few other mosquito species were captured, we lack information on the role of other mosquito species in the transmission of lineage 2 WNV [1].

Our study was a follow up of the previous study that took place in the Drienov wetland and recorded WNV genome in oral and cloacal swabs of free living birds [3].The results of our study were likely affected by a low number of examined vectors, so the presence of WNV in the respective locations cannot be excluded. Due to global warming, one can assume that an increase in the temperature and humidity will support the spreading of the vector. This indicates the need for surveillance of West Nile fever in the sensitive regions.

CONCLUSIONS

The positive finding of TBEV in the Dúbrava region indicates the occurrence of a natural focus. However, further studies in this location involving vectors and reservoirs are needed to confirm this hypothesis. The negative finding of WNV in the investigated area could be affected by several factors. The important ones include the influence of the climate on the population of mosquitoes in the relevant season and low number of examined vectors. Thus, one cannot guarantee the absence of this agent in the locations investigated, which opens a space for further investigation of the occurrence of WNV in mosquitoes in Slovakia.

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REFERENCES

1. **Bakonyi, T. E.,Ferenczi, K., Erdelyi, O., Kutasi, T., Csorgo, B., Seidel, H., et al., 2013:** Explosive spread of a neuroinvasive lineage 2 West Nile virus in Central Europe, 2008/2009. *Vet. Microbiol.*, 165, 61—70.
2. **Biernat, B., Karbowiak, G., Werszko, J., Stanczak, J., 2014:** Prevalence of tick-borne encephalitis virus (TBEV) RNA in *Dermacentor reticulatus* ticks from natural and urban environment, Poland. *Exp. Appl. Acarol.*, 64, 543—551.
3. **Csank, T., Bhide, K., Bencurova, E., Dolinska, S., Drzewniokova, P., Major, P., et al., 2016:** Detection of West Nile virus and tick-borne encephalitis virus in birds in Slovakia, using a universal primer set. *Arch. Virol.*, 161, 1679—1683.
4. **Grešíková, M., Nosek, J., 1967:** Isolation of tick-borne encephalitis virus from *Ixodes ricinus* ticks in the Tribeč region. *Bull. World Health Organ.*, 36, 67—71.
5. **Grešíková, M., Nosek, J., 1981:** *Arboviruses in Czechoslovakia* (In Slovak). 1st edn., Veda, Bratislava, 181 pp.
6. **Hubalek, Z., Halouzka, J., 1999:** West Nile fever – a reemerging mosquito-borne viral disease in Europe. *Emerg. Infect. Diseases*, 5, 643—650.
7. **Hubalek, Z., Ludvikova, E., Jahn, P., Treml, F., Rudolf, I., Svobodova, P., et al., 2013:** West Nile Virus equine serosur-

vey in the Czech and Slovak Republics. *Vector Borne Zoonotic Dis.*, 13, 733—738.

8. **ICTV, 2017:** *The ICTV Online (10th) Report on Virus Taxonomy*. Classification and Nomenclature of Viruses.
9. **Karbowiak, G., 2014:** The occurrence of the *Dermacentor reticulatus* tick, its expansion to new areas and possible causes. *Ann. Parasitol.*, 60, 37—47.
10. **Kerlik, J., Avdičová, M., Musilová, M., Dobler, G., Molčányi, T., Csank,T., et al., 2016:** Presence of tick-borne encephalitis virus in ticks from selected locations in Slovakia. In *Proceedings of the 5th scientific congress: Zoonoses — Protection of Public and Animal Health*, October 18—20, Slovak Medical University, Bratislava. 322 pp.
11. **Kunze, U., 2008:** Is there a need for travel vaccination against tick-borne encephalitis? *Travel Med. Infect. Dis.*, 6, 380—383.
12. **Rajčáni, J., Čiampor, F., 2006:** *Family Flaviviridae. Medical Virology* (In Slovak). Bratislava, Slovak Academy of Sciences, 436—451.
13. **Scaramozzino, N. J., Crance, M., Jouan, A., DeBriel, D. A., Stoll, F. O., Garin, D., 2001:** Comparison of flavivirus universal primer pairs and development of a rapid, highly sensitive heminested Reverse Transcription-PCR assay for detection of flaviviruses targeted to a conserved region of the NS5 gene sequences. *J. Clin. Microbiol.*, 39, 1922—1927.
14. **Wójcik-Fatla, A., Cisak, E., Zajac, V., Zwoliński, J., Dutkiewicz, J., 2011:** Prevalence of tick-borne encephalitis virus in *Ixodes ricinus* and *Dermacentor reticulatus* ticks collected from the Lublin region (eastern Poland). *Ticks and Tick-borne Diseases*, 2, 16—19.
15. **Zeller, H. G., Schuffenecker, I., 2004:** West Nile virus: an overview of its spread in Europe and the Mediterranean basin in contrast to its spread in the Americas. *Eur. J. Clin. Microbiol. Infect. Dis.*, 23, 147—156.

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