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Retrospective review and current knowledge on the occurrence of West Nile virus in mosquito vectors, reservoirs and hosts in Slovakia (Central Europe)

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Summary. – West Nile virus (WNV) is a mosquito-borne flavivirus (the genus *Flavivirus*) representing a medical and veterinary public health concern. Birds are the most important reservoirs. Culicine mosquitoes transmit WNV to vertebrate hosts (including horses and humans) and migratory birds play role in its long-distance transport. Slovakia is geographically localised at the crossroad of migration routes connecting South Europe and Africa with breeding localities in the Western, Northern, Central and Eastern Europe and Siberia. This review summarizes historical and present knowledge on WNV in Slovakia during a period of more than fifty years. Five European mosquito species capable to transmit WNV are native in Slovakia. Based on recent research results, the major role in the WNV transmission is attributed to *Culex* mosquitoes, which are also the most abundant species. Virus isolates from birds that succumbed to WNV infection are genetically close to Central European strains. Historical and recent results point out, that WNV circulates in the population of vectors, reservoirs and hosts for decades. Although West Nile fever epidemics in Slovakia were not reported yet, virus isolation, molecular detection and serological findings in reservoirs and hosts confirm that sporadic cases occur. Furthermore, the first autochthonous human case may indicate favourable conditions for WNV transmission to humans. The climate change and precipitation anomalies may favour to increase vector abundance, hence increase the chance of WNV epidemics. This review highlights an urgent need of a countrywide surveillance program aimed on the WNV occurrence in vectors and reservoirs in Slovakia.

Keywords: West Nile virus; flavivirus; vector-borne pathogen; arbovirus

Introduction

West Nile virus (WNV) is a mosquito-borne flavivirus (the genus *Flavivirus*, the family *Flaviviridae*). It belongs to the Japanese encephalitis serocomplex. Genetic analyses segregates WNV into two main lineages, the lineage 1 and the lineage 2. Lineage 1 strains are distributed in North Africa, Europe, North America and Australia. The lineage 2 comprises the original strains from sub-Saharan Africa, Madagascar and Europe. The lineage 3 includes the Rabensburg virus isolated in the Czech Republic (Bakonyi *et al*., 2005). The lineage 4 comprises of a strain isolated in the Caucasus, Russia (Lvov *et al*., 2004). Lineage 5, often designated as a separate clade 1c within the lineage 1 was isolated in India (Lanciotti *et al*., 2002). Several other lineages have been described, a putative lineage 6 from Spain (Vázquez *et al*., 2010); lineage 7, which strains were isolated from alternative hosts, like ticks and rodents in western

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Abbreviations: WNV = West Nile virus; ECDC = European Centre for Disease Prevention and Control; CDC = Centers for Diseases Prevention and Control; TBEV = Tick-borne encephalitis virus; YFV = Yellow fever virus; HI = hemagglutination-inhibition; USUV = Usutu virus; NAb = neutralizing antibodies; HIAb = hemagglutination inhibiting antibodies; μVNT = micro-titration virus neutralization test; ELISA = enzyme-linked immunosorbent assay; E = envelope; NS = non-structural protein

Africa (Fall *et al*., 2017). A putative lineage 8 was isolated in mosquito *Culex perfuscus* in Kedougu, Senegal (Fall *et al*., 2014) and finally also a putative lineage 9 isolated in Austria from mosquitoes *Uranotaenia unguiculata*, however, this one is also considered as a sublineage of lineage 4 (Pachler *et al*., 2014). WNV is one of the most widespread flaviviruses distributed in Africa, Asia, Europe and Australia. In 1999, WNV was introduced into the New World, and in the subsequent years it spread thorough the North and South Americas causing epidemics in human, horse and bird populations (Campbell *et al*., 2002).

In the rural cycle, WNV is maintained in a bird-mosquito-bird transmission cycle (Hubálek and Halouzka, 1999). Birds are the reservoirs, in which the long-term viremia reaches high levels. Mosquitoes become infected during blood meal on a viremic host and transmit WNV to birds or other vertebrates. For thorough review of WNV infection in vertebrate hosts the reader is advised to van der Meulen *et al*. (2005).

Since the first isolation of WNV from a febrile woman in Omogo (West Nile district; Uganda) in 1937 (Smithburn *et al*., 1940), sporadic cases and outbreaks of West Nile fever have been reported in Europe and the Mediterranean basin (Murgue *et al*., 2001a). Neurological disease after WNV infection was reported in horses and humans, but the main course of infection is asymptomatic or as a mild febrile illness (Murgue *et al*., 2001b; Campbell *et al*., 2002). In Eastern Europe, no large epidemic of West Nile fever has been reported; however, a large outbreak in Bucharest (Romania 1996) caused 352 human cases with symptoms of acute central nervous system infection, out of which seventeen died (Tsai *et al*., 1998). In 1997, the first human WNV infections were confirmed in South Moravia (Czech Republic) after floods and in the same year, the virus was isolated from mosquito pools (Hubálek *et al*., 1998, 1999). Further serological investigations in the bird population of South Moravia confirmed the circulation of WNV (Hubálek *et al*., 2008). In 2018, a total of five autochthonous human cases of West Nile virus infection were confirmed. One infected person received ambulatory treatment. Four patients were hospitalised, three of them did not develop complications. One patient died. It was 72-year-old woman from Břeclav district, unsuccessfully treated on suspected tick-borne encephalitis due to a preliminary serological diagnostics. Positive diagnosis for WNV was made post mortem based on retrospective blood and cerebrospinal fluid testing (Ciupek *et al*., 2019). In 2003 and 2004 in Hungary, two strains of WNV lineage 1 and lineage 2 caused lethal encephalitis in a flock of geese and a goshawk in south-eastern Hungary. During the outbreak in the geese flock, fourteen human cases were reported in the same area (Bakonyi *et al*., 2006). It was the first time when lineage 2 was reported in Europe.

The occurrence of WNV lineage 2 in Europe highlights the role of migratory birds in long-distance spread. The majority of bird species living in the seasonal environment of Europe undergo some type of periodical migration (Newton, 2008). European territory with its autochthonous avifauna is included in the western Palearctic Region and European breeding birds migrate mainly through western Palearctic-Afrotropical migration system (Berthold, 1993). About 250 bird species move through this migratory corridor two times per year (Newton, 2008). Hahn *et al*. (2009) assumed that 1.52-2.91 billion of European passerine and non-passerine birds cross the Sahara Desert each autumn. Mallards, ruffs and cranes migrating from Russia fly from eastern Palearctic region and form abundant clusters in Slovak territory, but small passerines originating from eastern Palearctic were also recorded in Slovakia (Gálffyová *et al*., 2010; Fulín *et al*., 2015). It can be assumed, that Central Europe is geographically situated at the crossroads of western and eastern Palearctic-Afrotropical migration routes connecting wintering places in South Europe and Africa with breeding localities in the Western, Northern, Central and Eastern Europe and Siberia (Klvaňa and Cepák, 2008; Fig. 1).

The present review summarizes historical and present knowledge on WNV in Slovakia in a period of more than fifty years. Resulting compilation of historical original articles from the last century is complemented by results of recent studies aimed on competent mosquito vector occurrence in Slovakia and their biology, the prevalence of WNV in populations of mosquitoes and vertebrate hosts in Slovakia.

West Nile virus in mosquitoes

Mosquitoes are the most important vectors of WNV. The virus is transmitted into the vector during blood meal on a viremic reservoir and later transmits the virus to a new host. In the nature, arboviruses also persist by overwintering. Recent study points on the role of hibernating *Culex pipiens* mosquitoes captured in Southern Moravia (Czech Republic) in the overwintering in Europe (Rudolf *et al*., 2017). Vertical transmission is another means that supports the circulation of vector-borne pathogens in the nature. Nelms *et al*. (2013) demonstrated vertical transmission of WNV under natural and laboratory conditions. However, viral load in the infected females seems to be crucial for vertical transmission of the virus to larvae and to adult progeny.

WNV was detected in 65 species of mosquitoes (Petersen *et al*., 2013), but in Europe successful isolation was reported from ornithophilic *Cx. pipiens*, *Cx. modestus* and *Coquillettidia richardii* mosquito species. As a secondary vector species of WNV were reported *Aedes cantans* and

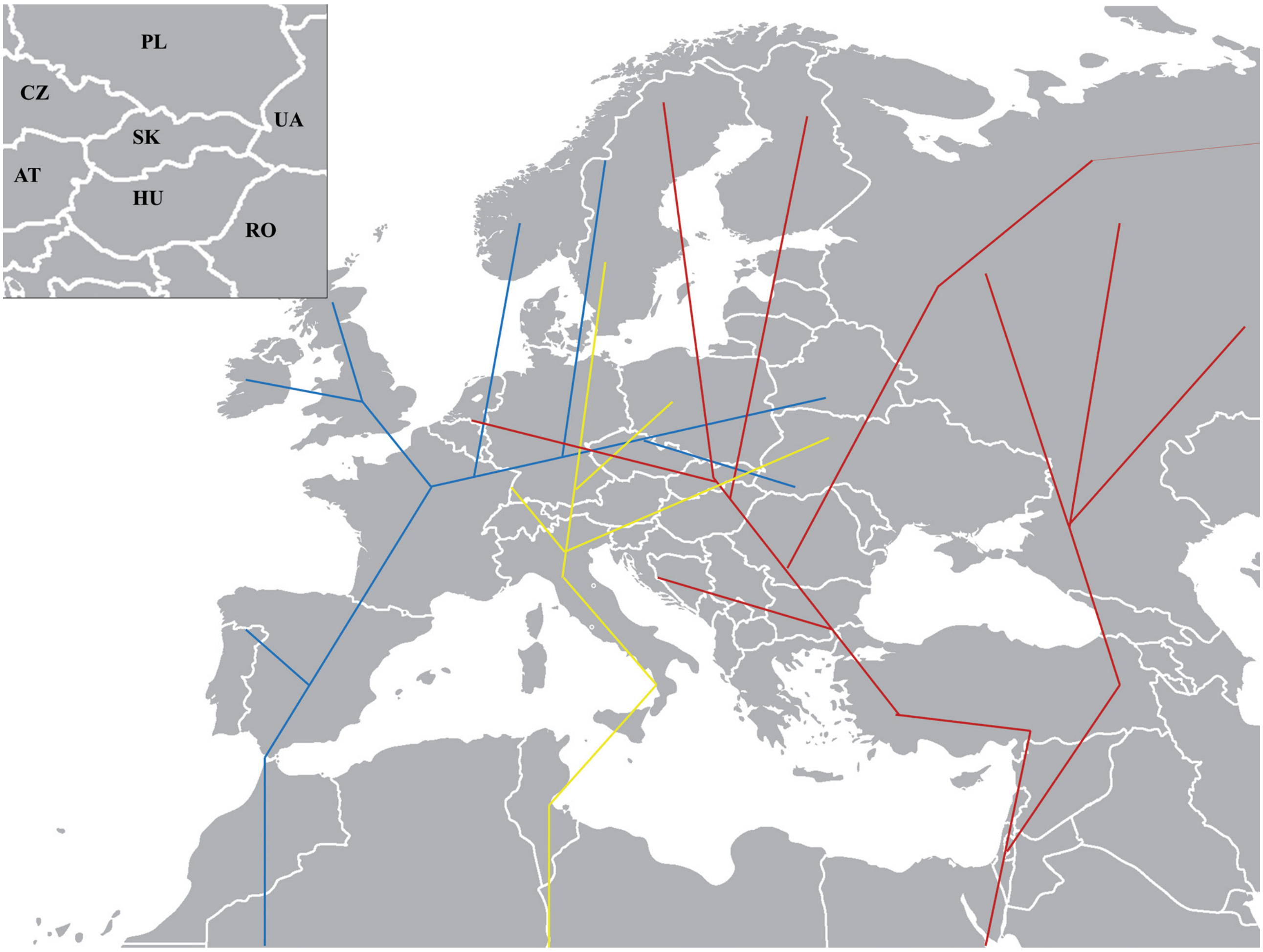

Fig. 1

Avian Palearctic-Afrotropical migration system

Legend: the complexity of European avian migration routes. Blue line – Palearctic western route from north-western, central and western Europe through Gibraltar into western, central and southern Africa. Yellow line – Palearctic central route from northern, central, western and southern Europe over the central Mediterranean to northern, central and southern Africa. Red line – Palearctic eastern route from western, central and north-eastern and eastern Europe through the Middle East to eastern and southern Africa. The insert depicts Slovakia and its neighbouring countries: SK – Slovakia, PL – Poland, UA – Ukraine, RO – Romania, HU – Hungary, AT – Austria and CZ – Czech Republic.

Anopheles maculipennis. It is assumed that some ticks, like *Hyalomma*, *Argas, Ornithodoros* and *Ixodes* possibly play role in the transmission of WNV in dry or urban ecosystems in Russia (Hubálek, 2008; Moskvitina *et al*., 2008).

Current research focuses on identification of mosquito species able to transmit WNV. These studies provide insight into the relationship of biotic and abiotic factors that may influence the WNV vector competence of these species. Such factors are temperature, origin and biotype of mosquitoes and laboratory conditions (Balenghien *et al*., 2007, 2008; Huber *et al*., 2014; Osório *et al*., 2014; Fortuna *et al*., 2015; Fros *et al*., 2015; Fritz *et al*., 2015; Blagrove *et al*., 2016; Vogels *et al*., 2016, 2017; Jansen *et al*., 2019).

The following part of the review focuses on WNV vector competence studies carried out on European mosquito species. Biological properties, distribution and the role in the spread of WNV of mosquitoes occurring in Slovakia are also discussed. Figure 2 depicts the localities in Slovakia, where WNV was isolated or detected in mosquitoes.

Vector competence of mosquitoes

There are almost 60 mosquito species in Slovakia, with activity season from middle of April to late October

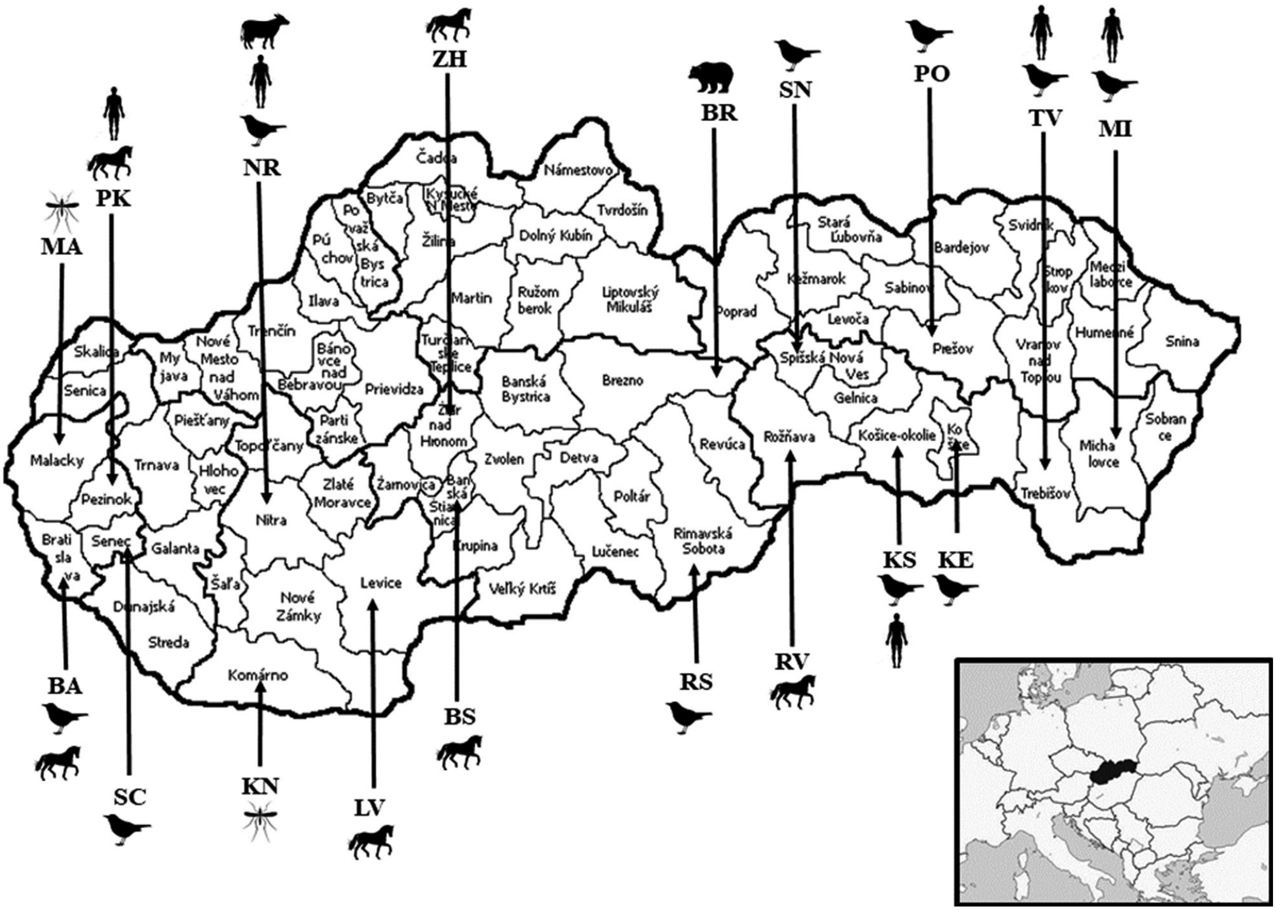

Fig. 2

Localities where WNV infections in vectors (mosquitoes) and hosts (birds, horses, cattle, bears, humans) were detected in Slovakia
Legend: The insert in the lower right corner depicts location of Slovakia in the central Europe. District abbreviations: BA – Bratislava; BS – Banská Štiavnica; KE – Košice; KN – Komárno; KS – Košice okolie; LV – Levice; MA – Malacky; MI – Michalovce; NR – Nitra; RS – Rimavská Sobota; RV – Rožňava; SN – Spišská Nová Ves; PO – Prešov, TV – Trebišov, ZH – Žiar nad Hronom. Wild living ruminants and wild boars were sampled in the Záhorská lowland, which stretches through Malacky and Senica districts in the western Slovakia.

(Strelková and Halgoš, 2012). The distribution of competent vectors plays a crucial role in a surveillance and a control of WNV. However, it needs to be mentioned that a countrywide surveillance program of mosquitoes is not establish in Slovakia. Records about species occurrence are from old data, mainly from the 60s and 80s of last century (Čepelák, 1984; Jalili *et al.*, 2000; Országh *et al.*, 2001) and from recent results of local research projects (Strelková and Halgoš, 2012; Bocková and Kočišová, 2016; Dzidová *et al.*, 2016; Oboňa *et al.*, 2017; Čabanová *et al.*, 2018, 2019).

So far, the transmission rate of WNV has only been measured in seven mosquito species occurring in Europe: *Aedes albopictus*, *Ae. caspius*, *Ae. detritus*, *Ae. japonicus*, *Culex modestus*, *Cx. torrentium* and *Cx. pipiens*. Except *Ae. japonicus*, six species occur in Slovakia (Jalili *et al.*, 2000; Strelková and Halgoš, 2012; Bocková and Kočišová, 2016). The transmission rate indicates whether the virus is present in the saliva of the vector and thus provides an overview of the proportion of mosquitoes capable of transmitting the pathogen. Transmission rate is the most important vector competence parameter since only mosquitoes with infectious saliva are capable of transmitting pathogens (Turell *et al.*, 2001; Balenghien *et al.*, 2008; Fortuna *et al.*, 2015; Fros *et al.*, 2015; Vogels *et al.*, 2016; Jansen *et al.*, 2019).

Vector competence, biology and the distribution of Culex mosquitoes in Slovakia

Vector competence

The main vectors of WNV in Europe are mosquitoes of the genus *Culex*, which use birds, horses, and humans as hosts. *Cx. pipiens* mosquitoes have been identified as one of the most important WNV vectors in the United States and Europe. A transmission rate of WNV via *Cx. pipiens* ranges from 0–60%. The maximum transmission rate of North European *Cx. pipiens* populations is 33%, while South European populations is 60% (Fortuna *et al.*, 2015; Fros *et al.*, 2015; Vogels *et al.*, 2016). These results can be partly explained by the difference in the vector competence between geographically distant populations. However, the most important limiting factor is temperature. Several European studies have confirmed that vector competence increases with the temperature. In the 18-28°C temperature range, the WNV transfer rate increased from 0% to 33%. For this reason, average North European temperatures (~18°C) appear to be an important limiting factor for WNV transmission (Fros *et al.*, 2015; Vogels *et al.*, 2016).

A mosquito biotype can be considered as a potentially important vector competence factor. Species *Cx. pipiens* includes two behaviourally different biotypes: *molestus* and *pipiens* (+ hybrids). While *Cx. pipiens pipiens* favours avian hosts, therefore, it is important in the natural WNV transmission cycle; *Cx. pipiens molestus* favours mammalian blood and along with hybrids, plays a role in the transmission of WNV from birds to humans (Fritz *et al.*, 2015; Osório *et al.*, 2014). So far, the results from research indicate that there is no difference in vector competence between *Cx. pipiens's* biotypes. However, both biotypes respond differently to temperature changes. Higher temperatures increased the transfer rate of *Cx. pipiens pipiens* and hybrids, but not the *molestus* biotype (Vogels *et al.*, 2016). Further research is needed to confirm the importance of the biotype in WNV transmission (Vogels *et al.*, 2016, 2017).

In addition to temperature and biotype, laboratory conditions can significantly affect WNV vector competence (virus strain, virus titer, incubation time and temperature). Jansen *et al.* (2019) compared the vector competence of both *Cx. pipiens* biotypes and *Cx. torrentium*. Mosquitoes were orally infected and kept at 18°C, 21°C, 24°C and 27°C for 14 or 21 dpi. All mosquitoes showed the presence of WNV in saliva, only at temperatures of 24°C and 27°C, and especially at an extended incubation period (21 days). The highest transmission rate of 17% and 24% was surprisingly measured in *Cx. torrentium* mosquitoes at 24°C and 27°C, respectively, while for *Cx. pipiens* biotypes the transmission rate was only 3%. *Cx. torrentium* was identified as a susceptible vector for WNV in Central and Northern Europe.

In the studies of Balenghien *et al.* (2007, 2008), vector competence of *Cx. modestus* for WNV transmission have been tested. A relatively high WNV transfer rate ranging from 40–55% have been observed (Balenghien *et al.*, 2007, 2008), but in both studies mosquitoes were exposed to high WNV titer (10^{10} PFU/ml). Thus, the WNV titer may explain the relatively high transfer rate (Vogels *et al.*, 2017).

Biology and the distribution of Culex mosquitoes in Slovakia

The major WNV vector, *Cx. pipiens* actually creates a complex of cryptic, morphologically not-distinguishable species and biotypes that can be differentiated only on molecular level (Smith and Fonseca, 2004; Becker *et al.*, 2010). Moreover, the differences between the members are not only genetic, but also ecological and behavioural what can change a perspective of a transmission route of the virus. From Slovak native mosquito fauna *Cx. torrentium* is supposedly strictly ornithophilic species occurring in man-made and natural habitats, often with the sibling species *Cx. pipiens sensu stricto* (Crantson *et al.*, 1987). Adult females of *torrentium* rest in extra-domestic habitats and overwinter successfully, hence imago can transmit WNV to the next season (Crantson *et al.*, 1987; ECDC 2013). The second member of the *pipiens* complex, *Cx. pipiens s. s.* is designed as two biotypes. The biotype *pipiens* is believed to be ornithophilic and can inhabit almost all kinds of water sources. This form occurs in urban and rural habitats and overwinters in cool hibernacula like cellars or caves (Crantson *et al.*, 1987; Becker *et al.*, 2010). On the contrary, the biotype *molestus* is well known to create a nuisance for a man, both outdoors and indoors, occupying principally an urban environment and can be found even in undergrounds, where it can shelter during winter. The form *molestus* is also feeding on birds and for this reason is considered to be a bridge vector of WNV (Becker *et al.*, 2010). However, according to recent data the seeking activity of all *pipiens* complex members is not so strict as was expected and the field studies suggest that all of them can serve as an infection bridge from birds to human (Börstler *et al.*, 2016). Moreover, a hybridization of the *pipiens/molestus* biotypes was observed and the hybrids have a high transmission rate of WNV (Fonseca *et al.*, 2004; Ciota *et al.*, 2013). Therefore, it is not surprising that the *Cx. pipiens* complex is one of the major problems in a mosquito taxonomy and the distribution of their members is miss-identified (Fonseca *et al.*, 2004).

The term *Cx. pipiens* complex will be used for the previous records of *Cx. pipiens s.s.* or *Cx. torrentium* from Slovakia due to above mentioned problematic identification. The *Cx. pipiens* complex members are one of the most distributed mosquitoes in Slovakia, active from early spring to late autumn. Mosquitoes of this complex are common in urban and rural habitats (e.g. Karlova Ves, Devínske Jazero, Vysoká pri Morave, Malacky – Rakáreň, Komárno, Pavlovce nad Uhom, Košické Oľšany) and natural habitats are situated in higher altitudes (Tatranská Lomnica, Tatranská Kotlina, Vyšné Hágy) (Čepelák, 1984; Jalili and Halgoš, 2005; Strelková and Halgoš *et al.*, 2012; Bocková *et al.*, 2013a; Dzidová *et al.*, 2016). The first molecular identification of the complex was provided in urban parts of Bratislava. The biotype *pipiens* was predominant and no occurrence of a hybridization and/or *Cx. torrentium* was found during the study (Čabanová *et al.*, 2018). Subsequent molecular research confirmed the *Cx. pipiens* form *pipiens* as the most abundant member of the *pipiens* complex in Slovakia, too. However, the second study revealed a hybridization in some Slovak regions and also DNA of *Cx. torrentium* was recognized (Čabanová, unpublished).

Another very important vector of WNV is *Cx. modestus* (Rudolf *et al.*, 2014). Its habitat preferences are stricter than for the *Cx. pipiens* members. *Cx. modestus* likes sunlit water area as swamps, marshes, ponds, irrigation channels or meadows which are likely to host a rich population of migratory and resident birds (Votýpka *et al.*, 2008). The species is active from a summer to beginning of an autumn. *Cx. modestus* is feeding on both, mammals and birds and it is causing a nuisance for humans in area of their occurrence (Votýpka *et al.*, 2008; Becker *et al.*, 2010). The species is described as common in Slovakia. A majority of the records were described in the Záhorská lowland and the Eastern Slovak lowland (Čepelák, 1984; Országh *et al.*, 2001), but it was also found as the second most abundant mosquito in fishponds and wetland areas of Komárno district (Čabanová *et al.*, 2019).

WNV vector competence, biology and the distribution of Aedes mosquitoes in Slovakia

Vector competence

The vector competence for WNV transmission was determined only for several species of the genus *Aedes*. Transmission rate in the Italian *Ae. albopictus* population varied from 0% to 40%. The authors attribute this significant percentage difference to the various extrinsic incubation periods used in the study (Fortuna *et al.*, 2015). Despite the proven vector competence, *Ae. albopictus* is not considered as a vector capable of spreading WNV. Birds that represent the most important WNV reservoirs are not preferred hosts for this mosquito species. *Ae. albopictus* preferred hosts are humans (dead-end hosts for WNV). Therefore, there is a low probability that mosquitoes will be infected by sucking blood from the amplification hosts (Muñnoz *et al.*, 2011). Other representatives of the genus *Aedes* with, at least the minimum transmission rate are *Ae. detritus* (~21%) (Blagrove *et al.*, 2016) and *Ae. caspius* (0–1%) (Balenghien *et al.*, 2008; Huber *et al.*, 2014). These studies suggest that mosquitoes of the genus *Aedes* do not contribute significantly to the spread of WNV to different parts of Europe (Vogels *et al.*, 2017).

The role of *Ae. japonicus* as a susceptible vector for WNV transmission in Europe is questionable. In several American studies was *Ae. japonicus* identified as a susceptible vector for WNV transmission, with virus reaching high titres (Sardelis and Turell, 2001; Turell *et al.*, 2001). However, German populations of *Ae. japonicus* are refractory to infection with WNV (Huber *et al.*, 2014). The differences in vector competence between European and American populations should be the subject of further research.

Biology and the distribution of Aedes mosquitoes in Slovakia

From native *Aedes* (*Ochlerotatus*) species, *Ae. caspius* and *Ae. detritus* can serve as vectors of WNV. The first one is recognized as a common species in Slovakia. *Ae. caspius* is a floodplain mosquito. In our conditions, the river valleys are the most favourable habitat for its occurrence. It rests mainly outdoors and feeds on animals and humans. *Ae. caspius* is able to fly long distances (up to 10 km) from its breeding site and it is resistant against heat and drought (Becker *et al.*, 2010). In Slovakia, this mosquito was recorded mainly in the Záhorská lowland, the Danube lowland, Beskydy and the Eastern Slovak lowland (Čepelák, 1984; Országh *et al.*, 2001; Bocková *et al.*, 2013a; Čabanová *et al.*, 2018).

Ae. detritus is a snow-melt mosquito breeding in a brackish, saline water. The species is feeding on humans, birds and livestock. It can fly indoors to find a host, but it rests in the vegetation around larval sites (Crantson *et al.*, 1987; Becker *et al.*, 2010). *Ae. detritus* inhabits coast lines, but it can be found also in freshwater wetlands (Becker *et al.*, 2010; Medlock and Vaux, 2015). To our knowledge, *Ae. detritus* was recorded only in one locality of the eastern Slovakia – Sobrance spa, characteristic with sulphur-saline mineral water (Čepelák, 1984; Országh *et al.*, 2001).

The potential risk of arboviral transmission may be created also by non-native species – *Ae. albopictus*. The invasive Asian tiger mosquito extensively colonized new regions in Europe via global transport and it is responsible for Chikungunya and Dengue outbreaks in Italy, France and Croatia (Vaux *et al.*, 2019). The species is able to breed in variable natural and artificial water areas as tree-holes,

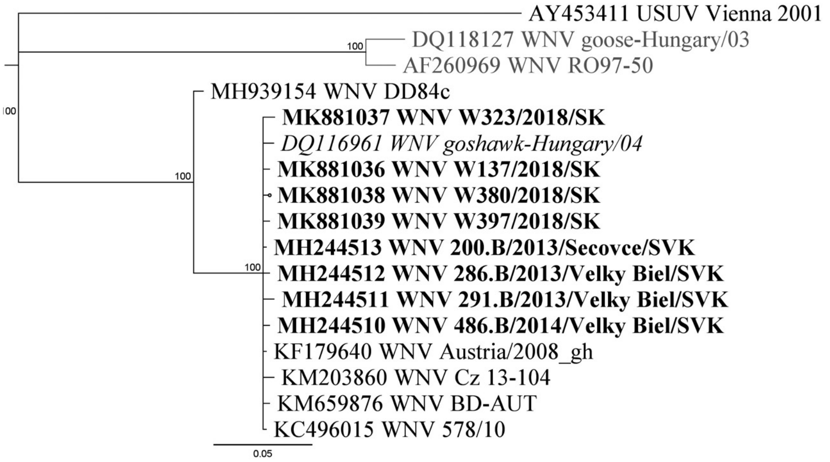

Fig. 3

Phylogenetic comparison of WNV strains detected in mosquitoes captured in Slovakia

Legend: Neighbor-Joining tree builder method with Tamura-Nei genetic distance model was used for phylogenetic tree assembly (Geneious version 9.1.8, Biomatters Ltd., New Zealand). A 279 bp long portion of the E protein gene of WNV isolates from Slovakia (bold) was compared with WNV lineage 1 (grey branches) and lineage 2 strains and USUV as outgroup. The reference strain is italicized.

rock holes, tyres, flower pots, even tin cans etc. The eggs of *Ae. albopictus* are very resistant what helps to their successful transport by vehicle tyres. Females are feeding on humans and mammals, occasionally on Passeriformes and Columbiformes birds (Schafner *et al*., 2004; Becker *et al*., 2010). *Ae. albopictus* was for a first time recognized in Slovakia in summer 2012. Four adult females were trapped in a rural habitat of Šebastovce village (near Košice city, the eastern Slovakia) (Bocková *et al*., 2013b). The current distribution of the species in Slovakia is unknown; however, records from neighbouring countries suggest a high risk for mosquito invasion (Šebesta *et al*., 2012; Rudolf *et al*., 2018).

WNV was detected also in another potential vectors. From our native mosquitoes, the virus was detected in *Coquillettidia richardii, Cx. territans, Cx. theileri, Ae. cantans, Ae. cinereus, Ae. dorsalis, Ae. excrucians, Ae. vexans, Ae. sticticus, Annopheles maculipennis* (Hubálek and Halouzka, 1999; Országh *et al*., 2001; Higgs *et al*., 2004; CDC, 2016).

WNV in vector population

Labuda *et al*. (1974) sampled female mosquitoes by a human landing catch in a wooden area of Malacky-Rakáreň in the western part of Slovakia. Virus isolation attempts were carried out by intracerebral infection of suckling mice and infection of the PS pig kidney cell line. One out of 129 pools killed the mice after 6 days in the first passage and subsequent passage shortened the incubation period to 4 days. In PS cell cultures, cytopathic effect appeared 4–5 days after inoculation. The pathogenic agent was designated as strain 99 and showed close antigenic relationship to WNV, TBEV and Yellow fever virus (YFV). In the hemagglutination-inhibition (HI) test, the immune serum against strain 99 reacted only with WNV, TBEV and YFV antigens in titres 1:1280, 1:40 and 1:80, respectively. Reactions with Sindbis virus, Western equine encephalitis virus, Ťahyňa and Čalovo viruses were all negative. The strain 99 antigen reacted with WNV, TBEV and YFV specific antisera in titres of 1:1280, 1:160 and 1:80, respectively. Complement fixation and neutralization tests also showed close relatedness or identity with WNV. *Ae. cantans* is a minor vector of WNV and the virus was detected in this species also in Ukraine and Bulgaria (Hubálek and Halouzka, 1999).

After that, no attention was paid to WNV vector surveillance in Slovakia for more than 40 years. In 2017, Mojžišová *et al*. (2017) provided a local screening for flaviviruses in mosquitoes trapped in Drienovec (Košice district) with negative result. In 2018, a mosquito sampling was conducted in two separated steps. The long-term sampling was provided in the suburban part of Komárno city, Nová Stráž by BG-Mosquitaire trap enriched by CO_2 (Biogents, Germany). The short-term, but more extensive part of the survey was focused on fishponds and wetlands in Komárno district. EVS (encephalitis vector survey) traps with dry ice were used. Female mosquitoes (n = 2817) were trapped, morphologically identified and screened for flaviviral nucleic acid. The minimum prevalence rate of WNV in pools was 0.46 % and all sequences were genetically related to WNV lineage 2 (Čabanová *et al.*, 2019; Fig. 3). It is noteworthy, that also Usutu virus (USUV) was detected in mosquitoes from Komárno district with a prevalence rate 0.25% (Čabanová *et al.*, 2019).

In years 2018 and 2019, mosquitoes were trapped also in other parts of Slovakia. Preliminary results from year 2018 confirmed circulation of flaviviruses in both, urban and rural habitats and indicate prevalence differences of the viruses' distribution between geographical areas of Slovakia. Surprisingly, USUV was much more prevalent in mosquitoes than WNV. Co-occurrence of both viruses was observed, as same as in a first study conducted in Komárno (Čabanová *et al.*, unpublished).

West Nile virus in vertebrate hosts

Migratory birds are suggested to play a role in long distance dissemination of WNV (Rappole *et al.*, 2006; Jourdain *et al.*, 2007). The most susceptible with possible clinical implications are corvids (Corvidae) (Komar *et al.*, 2003; LaDeau *et al.*, 2007). Other passeriform species, like thrushes (Turdidae), sparrows (Passeridae) and raptor and owl species (Accipitriformes, Falconiformes and Strigiformes) may shed large amounts of virus with infrequent clinical disease (Langevin *et al.*, 2005; Kilpatrick *et al.*, 2006; Nemeth *et al.*, 2006). Beside birds, WNV is able to infect a wide range of mammals, reptiles and amphibians. Clinical infection in these hosts are rare, but humans and equids are highly susceptible to WNV infection, what may lead to severe infection of central nervous system (van der Meulen *et al.*, 2005). This part of the review presents a summarization of information about WNV circulation in birds, free-living animals, cattle, horses and humans in Slovakia obtained from historical and recent studies. Figure 2 depicts the localities where WNV infection was detected either by serological or molecular approach in vertebrate hosts.

Serological findings on WNV infection in free-living birds

WNV infection of birds has been detected in pigeons (*Columba livia f. domestica*) caught in urban settings in Bratislava (Sékeyová *et al.*, 1976). In the first group (n = 83), acetone and heat-treated sera showed 35 hemaggentination-inhibition (HI) positivity with titres ranging from 1:10 to 1:80. However, neutralizing antibodies (NAb) has not been detected in duck embryonal cells. In the second group of pigeons (n = 50), sera has been treated with acetone and WNV HI antibodies (HIAb) were detected in 26% with titres 1:10–1:20. Pigeon sera in the third group (n = 150) were treated with acetone and kaolin, and WNV HIAb reached 4% prevalence with titres 1:20–1:40. Samples of the two latter groups were not confirmed by neutralization test, what might suggest that WNV antigen has high affinity to non-specific inhibitors of human and animal sera (Sékeyová *et al.*, 1976).

Recently, prevalence of WNV NAb in birds (n = 109) caught in Slovakia was studied using ELISA and microtitration virus neutralization test (μVNT). In this study, WNV antibodies were detected in 11.9% of birds and seropositivity was confirmed by VNT in 9.2% of birds involved. One Western marsh-harrier (*Circus aeruginosus*), three Northern goshawks (*Accipiter gentilis*), one osprey (*Pandion haliaetus*), one common buzzard (*Buteo buteo*), one peregrine falcon (*Falco peregrinus*), one golden eagle (*Aquila chrysaetos*) and one house sparrow (*Passer domesticus*) were seropositive (Csank *et al.*, 2018).

The results of surveillance for WNV infection in transmigrating and breeding small passerines of Drienovská wetland (Košice-okolie district), which is a regionally important hotspot for migratory birds (approximately 18000 birds mist-netted and marked using rings yearly) was the first serological screening of breeding ornithofauna of known origin in the Slovak Republic (Csank *et al.*, 2019). Captured birds (n = 154) were tested for WNV, USUV and Tick-borne encephalitis virus (TBEV) infection by simultaneous μVNT. Local breeding birds and hatched juveniles were sampled during a breeding season in 2017. WNV NAb were detected in 11.7% (n = 18) of birds. In addition to seropositive adult birds (transmigrants and local breeders), three juveniles were also positive for the presence of WNV NAb. None of the tested birds had USUV NAb, but one adult blackcap (*Sylvia atricapilla*) was positive for TBEV infection (Csank *et al.*, 2019).

Individual birds (n = 855) caught between 2013 and 2018 belonging to 26 families and eight orders were tested for WNV antibodies by ELISA or μVNT. Overall, WNV antibodies were present in 4.8% (41/855). Seroreactors were from 3 orders (Accipitriformes, Ciconiiformes and Passeriformes), 11 families and 15 species. The species were the following: Northern goshawk (*Accipiter gentilis*, n = 4/6); marsh harrier (*Circus aeruginosus*, n = 1/1); white stork (*Ciconia ciconia*, n = 1/1); reed warbler (*Acrocephalus scirpaceus*, n = 1/15); Eurasian jay (*Garrulus glandarius*, n = 1/4); hawfinch (*Coccothraustes coccothraustes*, n = 4/60); chaffinch (*Fringilla coelebs*, n = 1/22); Eurasian river warbler (*Locustella fluviatilis*, n = 1/1); European robin (*Erithacus rubecula*, n = 9/91); great tit (*Parus major*, n = 3/167); common starling (*Sturnus vulgaris*, n = 1/9); blackcap (*Sylvia atricapilla*, n = 6/199); common whitethroat (*Sylvia communis*, n = 2/13); song thrush (*Turdus philomelos*, n = 4/37) and blackbird (*Turdus merula*, n = 2/60) (Csank and Korytár, not published). In a similar study carried out between

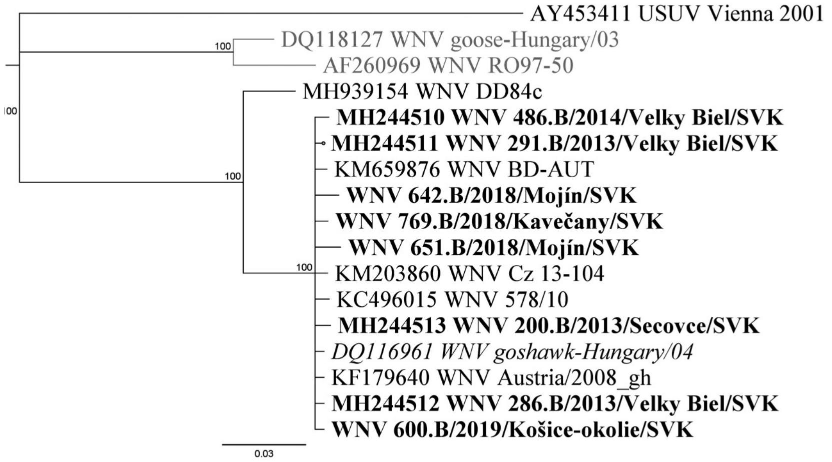

Fig. 4

Phylogenetic comparison of WNV strains isolated from dead birds in Slovakia

Legend: Neighbor-Joining tree builder method with Tamura-Nei genetic distance model was used for phylogenetic tree assembly (Geneious version 9.1.8). A 502 bp long portion of the NS5 gene of isolates from Slovakia (bold) was compared with WNV lineage 1 (grey branches) and lineage 2 strains and USUV as outgroup. The reference strain is italicized.

2004 and 2006 in Southern Moravia, 3.3% (13/391) of tested wild birds were seropositive for WNV antibodies. These belonged to 11 species: common coot (*Fulica atra*), common kingfisher (*Alcedo atthis*), reed warbler (*Acrocephalus scirpaceus*), sedge warbler (*Acrocephalus schoenobaenus*), marsh warbler (*Acrocephalus palustris*), Savi's warbler (*Locustella luscinioides*), reed bunting (*Emberiza schoeniclus*), blackcap (*Sylvia atricapilla*), penduline tit (*Remiz pendulinus*), blue tit (*Cyanistes caeruleus*) and common starling (*Sturnus vulgaris*) (Hubálek *et al.*, 2008).

WNV isolates from birds

Historical data reports on the isolation of several strains come from the blood and brain of green sandpiper (*Tringa ochropus*), black-headed gull (*Chroiocephalus ridibundus*), Northern lapwing (*Vanellus vanellus*) and European turtle dove (*Streptopelia turtur*) captured in 1972-73 at the Ipeľ, Rimava and Bodva rivers in south Slovakia. Three of them were designated as E13, E26 and E30 (Grešíková *et al.*, 1975; Ernek *et al.*, 1977). Antigens from these strains showed close relationship and reacted with WNV immune serum in equal titres as the homologous one. WNV was also isolated from black-headed gull arriving to south eastern Slovakia from their winter quarters (Ernek *et al.*, 1977). Unfortunately, there are no genetic analyses available for strains isolated in the past.

A recent study on detection of WNV in free-living birds by RT-PCR was carried out by Csank *et al.* (2016). One-hundred and ten birds, mainly passerines, were caught and swabbed from the oral cavity and cloaca. These birds were mist-netted on three localities: Drienovec (Košice-okolie district), Zajačia valley (Levice district) and Zemplínska Teplica (Trebišov district). Bird brains were collected from carcasses after accidents and after deaths in wildlife rehabilitation centres. Forty-five brains of raptors and small passerines originating from districts Nitra, Spišská Nová Ves, Košice-okolie, Košice and Trebišov were examined. The presence of WNV was detected in 21.8% (n = 24) of swabs in birds of 10 species and 17.8% (n = 8) brain samples of five avian species. The only samples suitable for virus isolation were the brain samples (Csank *et al.*, 2016).

Four WNV strains were isolated in Vero E6 cell line from four brain samples, three from Northern goshawks (*Accipiter gentilis*) and one from Eurasian sparrow hawk (*A. nisus*). All raptors died during clinical offset of WNV infection in 2013 and 2014. This is the first evidence of West Nile fever in reservoirs in Slovakia. Strains were designated as WNV 486.B/14/Velky Biel/SVK, WNV 291.B/13/Velky Biel/SVK, WNV 286.B/13/Velky Biel/SVK and WNV 200.B/2013/Sečovce/SVK (accession numbers MH244510–MH244513). The former three birds were from southwest Slovakia (Senec district) and the later from southeast Slovakia (Trebišov district). Genetic analysis showed close clustering with central European WNV lineage 2 strains detected in Hungary, the Czech Republic, Austria and Serbia (Fig. 4). Whole genome pairwise identity of the Slovak strains and the strains from the neighbouring countries ranged from 99.5–99.8 %. The comparison of virus polyproteins with the reference WNV lineage 1 and lineage 2 strains showed seven amino acid substitutions in structural (PrM$_3$, E$_{159}$

and E_{231}) and in non-structural ($NS1_{109}$, $NS5_{259}$, $NS5_{310}$ and $NS5_{600}$) proteins. These were further studied by comparison with polyprotein sequences of WNV strains isolated all over the world (n = 1903). Unique amino acid substitutions Phe_3 in the PrM protein and Ser_{231} in the E protein were common only with the lineage 7 Koutango strain, isolated from *Rhipicephalus guilhoni* tick in Senegal. Further research could reveal whether these substitutions influence the biological properties of WNV, including virulence and neuroinvasiveness (Drzewnioková *et al*., 2019).

In 2018 and 2019, four WNV strains were isolated (642.B/2018/Mojín/SVK, 651.B/2018/Mojín/SVK, 769.B/2018/Kavečany/SVK and 600.B/2019/Košice-okolie/SVK) in Vero E6 cell line from raptor brain samples (Csank *et al*., not published). Two strains were isolated from the brains of a three-year-old female and an eight-year-old male Northern goshawk from Mojín village (Rimavská Sobota district) in the southcentral Slovakia. Both birds showed weakness of legs, were not able to eat, went blind, lost coordination and died in late September and early October in 2018. The third WNV strain was isolated from the brain of a great grey owl (*Strix nebulosa lapponica*). The animal lived for two years in the Košice Zoological garden (Kavečany, Košice district) after the clinical phase lasting for several days, the owl died at the end of August 2018. In September 2019, a Northern goshawk was found at roadside in the Košice-okolie district. The bird was exhausted and shortly after admission to the University Veterinary Hospital (University of Veterinary Medicine and Pharmacy in Košice) died. Sequence analysis of the partial NS5 gene showed WNV lineage 2 clustering (Csank *et al*., not published) (Fig. 4).

WNV infection in cattle and wild animals

Cattle serum samples (n = 93) collected in 1965 in Horné Lefantovce (Nitra district) have been tested for antibodies against different arboviruses (Sékeyová and Grešíková, 1967). Here, eight serum samples contained WNV HI antibodies in titres ranging from 1:20-1:80, but three samples cross-reacted with TBEV and YFV antigens. One WNV seropositive sample with titre 1:20 cross-reacted with TBEV antigen, but TBEV HI antibody titre (1:320) was indicative for TBEV infection. In two cases, the infection could not be differentiated from TBEV or YFV.

Sera from hunting game and farm animals (n = 1505) collected during 1969–72 in Záhorská lowland were investigated for the presence of WNV NAb (Kožuch *et al*., 1976). Seroreactors were present only in hunting game (2%) in titres ranging 1:4–1:16. Seropositivity in wild-living ruminants was 4.2% (8/190) : 4.6% (3/65) of roe deer (*Capreolus capreolus*), in 8.3% (2/24) of fallow deer (*Dama dama*), in 3% (3/101) of red deer (*Cervus elaphus*). In wild boars (*Sus scrofa*) and European hares (*Lepus europaeus*) WNV NAb were present in 2.6% (1/38) and in 1% (6/596), respectively. Conversely, no antibodies were detected in cattle (n = 335) and calves (n = 46).

Wild-living artiodactyls were examined for WNV NAb in Břeclav district in South Moravia (Czech Republic). The animals were sampled in the years 1999–2008. Overall, 5.9% (36/611) of ruminants were positive: 4.8% (5/105) of roe deer, 4.1% (6/148) of red deer, 6.3% (18/287) of fallow deer and 9.9% (7/71) of mouflons (*Ovis musimon*) and 4.1% (17/412) of wild boars (non-ruminant species) (Hubálek *et al*., 2017).

WNV infection was detected also in bears (Madić *et al*., 1993; Farajollahi *et al*., 2003; Bronson *et al*., 2014). Vitásková *et al*. (2019) examined Eurasian brown bear serum samples (n = 24) sampled between 2011 and 2015 for antibodies against different viruses, including WNV. They found WNV antibodies in one bear (4 %) by ELISA sampled in the Low Tatras (Fig. 2). The serum was further tested in simultaneous virus neutralization and the titer of WNV NAb was 1:256 without cross-reaction with TBEV (Vitásková *et al*., 2019).

WNV infection in horses

Horses (n = 145) with and without travelling history abroad Slovakia were examined for WNV infection (Csank *et al*., 2018). Most of them originated from western Slovakia, where endemic transmission of WNV was recorded (Hubálek *et al*., 2013). In the group without travelling abroad Slovakia, 16.6% of horses presented WNV NAb. These animals were from Bratislava, Pezinok and Žiar nad Hronom districts. Overall 7% of horses with travelling history were seropositive. In this study, an equine WNV infection on the locality Kremnické Bane without previously known WNV circulation was confirmed. Six years old male horse without travelling history abroad Slovakia was tested seropositive for WNV NAb. The rest of horses from the same stables were seronegative. The horse didn't show any neurological symptoms. An overcome infection was diagnosed in this animal due the absence of IgM and due the high titer (1:320–1:640) of WNV NAb (Csank *et al*., 2018). It was suggested that the horse became infected in the region of Kremnické Bane. However, in personal communication the owner claimed a several weeks long period in summer in Cabaj-Čápor village in the Nitra district.

The first outbreak of West Nile fever in Hungary occurred in 2008 between August 26 and October 10 in 17 horses with neurological signs as ataxia, weakness, asymmetric gait, muscle tremor, hypersensitivity, cranial nerve deficits and recumbency (Kutasi *et al*., 2011). Twelve of them survived. WNV was detected by RT-PCR in the brainstem and lumbar region of the spinal cord of two horses and phylogenetic tree showed lineage 2 grouping.

Up to date, there is no information about an epidemic in horses in Slovakia.

WNV infection in humans

Human blood sera (n = 297) of healthy population were collected during 1962-64 from localities of western – Bratislava (Bratislava district; n = 75) and Jarok (Nitra district; n = 102); and eastern – Michalovce (Michalovce district; n = 120) Slovakia (Grešíková and Sékeyová, 1967). Specific antibodies against alphaviruses, bunyaviruses and flaviviruses have been screened by HI test. None of the samples were positive for HIAb against alphaviruses, Čalovo strains of Batai virus and Dengue virus 1. Several serum samples were positive against Ťahyňa and TBEV. No positive WNV samples were detected in Bratislava, but 2% of serum samples from Jarok and 3 % from Michalovce (n = 120) contained specific antibodies. In the group of serum samples from Jarok, one sample contained WNV HIAb with titre 1:20 and reacted only with WNV antigen. The second seropositive sample contained antibodies with titre 1:40, but cross-reacted with YFV with titre 1:20 (Grešíková and Sékeyová, 1967).

Most recently, a serologic survey was conducted using 464 human serum samples screened for WNV antibodies by ELISA. Three serum samples (0.65%) were positive for WNV NAb in ELISA. Due to the small volume, only two of the samples could be further examined by simultaneous µVNT. There was no cross reaction with TBEV antibodies. In one case (61-year-old woman from Košice-okolie county), the infection could not be distinguished from USUV, because the difference of geometric mean titers (GMT) was not at least 4-fold. In the second case (76-year-old woman; Kráľovský Chlmec district), GMT difference was 5.7 in the favour of WNV. Both women were hospitalized at the Department of Neurology due to monoparesis of the upper extremity, vertigo; both had a significant epidemiological history with frequent tick and mosquito bites and stay in an endemic region abroad Slovakia. Unfortunately, paired serum samples were not available (Dorko *et al.*, 2018).

The first confirmed autochthonous case of West Nile fever was diagnosed in the late summer of 2019. The man was infected after several mosquito bites in Slovenský Grob village (Pezinok district). The clinical description consists of mild, flu-like symptoms characteristic mainly by a fever and rush. The infection was confirmed by several methods, both serological and molecular approaches. The sequences were genetically related to WNV lineage 2 (Čabanová *et al.*, not published). Up to date, there is no official report on clinical West Nile fever cases or outbreaks in humans in Slovakia.

In the neighbouring Hungary, year 2018 was extraordinary in the number of autochthonous clinical human West Nile fever cases (Nagy *et al.*, 2019). Two hundred and fifteen WNV infections were notified and most of them with neurological symptoms. WNV lineage 2 strains were detected what correlates with the previous years (Nagy *et al.*, 2016).

Conclusions

WNV is one of the most distributed arboviruses with public health significance from medical and from veterinary point of view. Since its first isolation, WNV caused several outbreaks in human and animal population in the Old and New World. Results of historical and recent research point out, that WNV is endemic in Slovakia and circulates between its natural vectors – mosquitoes and hosts – birds, horses and humans for decades.

A majority of infected mosquitoes belongs to the *Cx. pipiens* complex. Furthermore, members of the complex are one of the most common and widely distributed mosquito species even in highly populated city area, what may highly obstruct a WNV control. WNV antibodies were detected in reservoirs, in horses and humans in different localities. The virus was isolated from vectors and dead reservoirs. The isolates are genetically close to central European WNV lineage 2 strains. The origin of seropositive samples and successful virus isolation attempts show that WNV is endemic along the south border of Slovakia. However, West Nile fever epidemics in humans and animals in Slovakia were not reported up to date, but sporadic cases occur. The epidemiological situation of WNV in Europe, the climate change and precipitation anomalies may favour to increase competent vector abundance or to establish an invasive mosquito species population, hence increase the chance of not only WNV epidemics.

A mosquito surveillance and control are one of the most important strategies against mosquito-borne diseases, especially when drugs and vaccines are not available (EMCA – European Mosquito Control Association/WHO, 2013). Targeted surveillance of the virus in mosquitoes can reveal epidemics in animals and humans and may help to focus on effective control measures. Mosquito surveillance provided by public health authorities, as is common in other European countries, is absent in Slovakia. Hence, there is a need for a comprehensive and countrywide program aimed on WNV surveillance in vectors and reservoirs.

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