Monitoring of West Nile Virus in Mosquitoes Between 2011–2012 in Hungary

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Abstract

West Nile virus (WNV) is a widely distributed mosquito-borne flavivirus. WNV strains are classified into several genetic lineages on the basis of phylogenetic differences. Whereas lineage 1 viruses are distributed worldwide, lineage 2 WNV was first detected outside of Africa in Hungary in 2004. Since then, WNV-associated disease and mortality in animal and human hosts have been documented periodically in Hungary. After the first detection of WNV from a pool of *Culex pipiens* mosquitoes in 2010, samples were collated from several sources and tested in a 2-year monitoring program. Collection areas were located in the Southern Transdanubium, in northeastern Hungary, in eastern Hungary, and in southeastern Hungary. During the 2 years, 23,193 mosquitoes in 645 pools were screened for WNV virus presence with RT-PCR. Three pools were found positive for WNV in 2011 (one pool of *Ochlerotatus annulipes* collected in Fényeslitke in June, one pool of *Coquillettidia richiardii* collected in Debrecen, Fancsika-tó, in July, and one pool of *Cx. pipiens* captured near Red-Footed Falcon colonies at Kardoskút in September). The minimal infection rate (MIR = proportion of infected mosquitoes per 1000 mosquitoes) of all mosquito pools was 0.25, whereas the MIR of infected species was 2.03 for *O. annulipes*, 0.63 for *C. richiardii*, and 2.70 for *Cx. pipiens*. Molecular data have demonstrated that the same lineage 2 WNV strain has circulated in wild birds, horses, humans, and mosquitoes in Hungary since 2004. Mosquito-based surveillance successfully complemented the ongoing, long-term passive surveillance system and it was useful for the early detection of WNV circulation.

Key Words: West Nile virus—Mosquito—Hungary—*Ochlerotatus annulipes*—*Coquillettidia richiardii*—*Culex pipiens*—Minimal infection rate.

Introduction

WEST NILE VIRUS (WNV) IS ONE OF THE most widely distributed mosquito-borne flaviviruses (Rossi et al. 2010, Weissenböck et al. 2010). WNV belongs to the Japanese encephalitis virus complex within the genus *Flavivirus* (family *Flaviviridae*) and has been further classified into a number of distinct lineages. Lineage 1 is present in Africa, Australia, India, and parts of Eurasia and has been responsible for outbreaks in the Americas since 1999, whereas lineage 2 strains have been known to exist only in sub-Saharan Africa until recently (Lanciotti et al. 1999, Hayes

2001, Zeller and Schuffenecker 2004). The first proven cases of lineage 2 virus infections outside of Africa were a Sparrowhawk and a Goshawk found dead in Hungary in 2004 (Erdélyi et al. 2007). Since then, lineage 2 WNV has become enzootic, and infections have been documented to cause illness and death in wild birds, horses, and humans in Central Europe, especially in Hungary and Austria (Bakonyi et al. 2006, Krisztalovics et al. 2008, Bakonyi et al. 2013). In the past 3 years, viruses belonging to lineage 2 have appeared in Greece (Chaskopoulou et al. 2011, Papa et al. 2011), Italy (Bagnarelli et al. 2011, Savini et al. 2012), Russia (Platonov et al. 2008, 2011), and Romania (Sirbu et al. 2011). A

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phylogeographic reconstruction of different lineage 2 strains showed that the European subcluster most probably originated in Hungary and then spread to Austria before reaching Greece and Italy (Bakonyi et al. 2013, Ciccozzi et al. 2013). More recently, another distinct lineage 2 WNV strain has been introduced and established outside of Africa, especially in Russia and Romania (Ciccozzi et al. 2013). Furthermore, serological evidence of WNV infections in horses has been demonstrated recently in Serbia and Croatia (Lupulovic et al. 2011, Barbić et al. 2012) along with direct detection of WNV in wild birds and outbreaks of human cases (Petrović et al. 2013a, b).

In general, the enzootic cycle of WNV is maintained between various reservoir bird species that amplify the virus and the biological vectors, which are mainly mosquitoes feeding on birds that occasionally transmit WNV to mammals (Savage et al. 1999, Apperson et al. 2004). WNV is transmitted by at least 64 mosquito species with diverse ecology and behavior (Centers for Disease Control and Prevention 2012). In Europe, the principal vectors are *Culex* species (i.e., *Cx. pipiens*, *Cx. modestus*), and *Coquillettidia richiardii* (Hayes 1989, Higgs et al. 2004, Lvov et al. 2004, Zeller and Schuffenecker 2004), which are also very abundant in some biotopes in Hungary. In a study at the Danube Delta (a region with enormous populations of resident and migrant birds and high number of mosquitoes) in 2008, 99% of the >10,000 captured mosquitoes representing 17 species belonged to five species. The most abundant ones were *Cx. pipiens* (44%), *Cx. torrentium* (27%), *Cx. modestus* (11%), *C. richiardii* (14%), and *Anopheles maculipennis* (3%). The two species most commonly captured on humans were *Cx. modestus* (35%) and *C. richiardii* (34%) in the same area (Reiter 2010). Thus, some species serve as enzootic vectors and maintain the infection among competent hosts, whereas others are bridge vectors between the reservoir avian species and the incidental dead-end hosts, as they usually feed on

birds but occasionally also on humans, resulting in a complex relationship between vectors and hosts (Medlock 2007, Gray and Webb 2014). The introduction of WNV lineage 2 to Hungary could most likely be attributed to long-distance migratory birds overwintering in central and southern Africa (Ciccozzi et al. 2013). However, the establishment and spread of WNV in Europe requires the presence of both amplifying bird species and transmitting arthropod vectors. The identification of different mosquito species serving as WNV vectors is an important task with direct implications for understanding WNV ecology and implementing adequate preventive measures, including effective vector control. This study aimed to identify the spectrum of potential mosquito vectors involved in the transmission of WNV in Hungary.

Materials and methods

Mosquito trapping and identification

Each year the main season of collection was from May to September, corresponding to the maximum annual activity of mosquito vectors. The sampling was performed by three teams at 24 locations within five geographic areas (Fig. 1). These collection areas were located in the Southern Transdanubium (four locations in Baranya county, area I), in northeastern Hungary (five locations in Szabolcs-Szatmár-Bereg county, area II), in eastern Hungary (six locations in Hajdú-Bihar county, area III), in southeastern Hungary (four locations in Békés county, area IV), and two locations in central Hungary (area V). In more detail, the mosquito trapping sites were as follows: In area I—marshlands near Pécs (22), bird ringing station in Sumony (24), dead channels of the Danube river near Mohács (21), the floodplains of Drava river near Drávaszabolcs border crossing point (23); in area II—floodplains of the River Tisza (two locations, 1 and 2), marshlands (two locations, 4 and 8), and a reservoir lake near

FIG. 1. Origin of the mosquito pools tested for WNV. The points identify the localities (open circles, localities without WNV positive pools; filled circles, localities with at least one positive pool). 1, Fényeslitke; 2, Szabolcsveresmart; 3, Döge; 4, Kékcse; 5, Kisvárd; 6, Anarcs; 7, Ajak; 8, Gyulaháza; 9–13, Debrecen; 14, Hortobágy-Halastó; 15, Egyek (HNP); 16, Dévaványa; 17, Körösladány; 18, Kardoskút; 19, Ócsa; 20, Felsőerek; 21, Mohács; 22, Pécs; 23, Drávaszabolcs; 24, Sumony.

Kisvárdá (5); in area III—gallery forests (two locations, 9 and 10), fishing ponds (two locations, 11 and 13) near Debrecen, a sampling site in the town center (12), and a sampling site in the Hortobágy National Park (HNP), which is a mosaic of alkaline marsh and steppe (15); in area IV—two locations at Kardoskút, near a Red-footed Falcon colony and a sheep stable (18), pigeon loft at Körösladány (17), poultry housing and pigsty at Dévaványa (16); in area V—cellars at Ócsa (19) and near a duck farm at Felsőerek (20). Additionally, sampling of overwintering mosquitoes was performed at locations near the above-mentioned sites in poultry stables (3), cellars (5, 7, 8), sheep barns (6, 8), and cattle stables (14).

In area I, adult mosquitoes were collected by updraft box traps with light attractant at the marshlands near Pécs and by human landing collection with an aspirator at every sampling site (Drávaszabolcs, Mohács, Sumony). During the sampling period, the traps were checked every day, and every fifth day the mosquitoes were removed from the traps, then immediately transported to the laboratory and killed at -20°C . Furthermore, at every sampling location mosquitoes were collected by human landing collection (landing mosquitoes were collected from the body surface for 15 min, after sunset) once a week and were transported to the laboratory alive and then killed at -20°C .

In areas II and III, surveys (bite counts and phenology examinations) were performed twice a month by a pooter (powered aspirator) when landing mosquitoes were collected from the body surface for 15 min after sunset. In wintertime, overwintering mosquitoes were collected either by mouth aspirator or pooter from the inner walls of buildings, cellars, and stables (horse, sheep, cattle, and poultry) at seven settlements (six near Kisvárdá, one in the HNP). After collection, the imagoes were stored in a cool box with ice pack until they were frozen in the laboratory.

In areas IV and V, sampling was performed by aspirator during several 1-day field trips. Mosquitoes were collected from the inner walls of buildings at Kardoskút (sheepfolds, cellar), Körösladány (pigeon loft, cellar), Dévaványa (poultry housing, pigsty), Ócsa (cellars), and by additional light trapping at Felsőerek and Kardoskút.

Mosquitoes were identified with a stereomicroscope according to the morphological identification keys of Becker et al. (2003) and Kenyeres and Tóth (2008). Mosquito identification was performed on cool pads providing low temperature. The specimens were grouped by species, sex, collection site, and date, and finally pooled with a maximum size of 50 individuals. Three different types of pools were distinguished based on the number of mosquitoes collected at a particular time and place. According to this system, pools of one to three mosquitoes, four to 19 mosquitoes, and 20–50 mosquitoes were created. Mosquito pools were placed in 1.5-mL microcentrifuge tubes and stored at -80°C until molecular detection of WNV.

Detection of WNV in mosquito pools

The pools of insects were homogenized in sterile phosphate-buffered saline (PBS) with a semiautomatic Tissue Lyser device (Qiagen, Hilden, Germany) followed by centrifugation at $10,000 \times g$ for 10 min. Viral RNA was extracted from 100 μL of the supernatant of mosquito pools using the automated Xtractor Gene nucleic acid extraction robot (Corbett Robotics

Pty. Ltd., Queensland, Australia) and the Total RNA Isolation Kit, Nucleospin 96 RNA (Macherey-Nagel, Düren, Germany). The samples were tested with a TaqMan technology-based real-time RT-PCR targeting the NS3 (Bakonyi et al. 2013) and the 5'-untranslated region (UTR) (Linke et al. 2007) coding regions of the WNV genome. The assay described previously by Bakonyi et al. (2013) was modified with a 3' black hole quencher (BHQ) and optimized as follows: 2.5 μL of template RNA was mixed with 1 μL each of forward and reverse primers (10 μM), 0.5 μL of probe (10 μM), 12.5 μL Reaction Mix buffer (Invitrogen, USA), 0.25 μL RNasin (Promega, USA) RNase inhibitor, and 0.5 μL SuperScript III RT/Platinum Taq Mix (Invitrogen, USA) in 6.75 μL of RNase-free water. Reaction mixture was heated to 48°C for 15 min for the reverse transcription, followed by 2 min at 95°C for the activation of the Taq polymerase and template denaturation. The cycling conditions were as follows: Amplification of the target cDNA in 45 cycles repeating denaturation of DNA at 95°C for 15 s and combined annealing and elongation at 60°C for 30 s. Fluorescence was detected at the annealing/elongation step. Positive samples were further confirmed by sequencing of the real-time PCR product in the case of positive mosquito pools.

Results

A total of 23,193 mosquitoes were collected and screened during 2011–2012 (Table 1). The composition of mosquito assemblages differed by study area. In area I, the most abundant mosquito was *Aedes vexans* (8,603/19,615, 43.9%) followed by *Ochlerotatus sticticus* (5,656/19,615, 28.8%) and *Ae. cinereus* (2,206/19,615, 11.2%). The most common species in area were *Ae. vexans* (814/1,723, 47.2%), *C. richiardii* (313/1,723, 18.2%), *Cx. modestus* (255/1,723, 14.8%), and *Ae. cinereus* (202/1,723, 11.7%). In area III, *C. richiardii* (229/601, 38.1%) and *O. cantans* (221/601, 36.8%) were the most abundant species near Debrecen, and *C. richiardii* (353/737, 47.9%), *Anopheles hyrcanus* (177/737, 24.0%), and *Cx. modestus* (96/737, 13.0%) at the site of HNP. In area IV, the most common species were *Cx. pipiens* (178/350, 50.9%) and *A. maculipennis* (166/350, 47.4%). In area V, the majority of the collected mosquitoes were captured during the winter period, resulting in a comparatively smaller number of samples. The most commonly found overwintering species were *Cx. pipiens* (78/135, 57.8%) and *Culiseta annulata* (50/135, 37.0%). In areas II and III, we analyzed the proportion of the two *Culex* biotypes (*pipiens* and *molestus*) and the result was near 1:1, which is in contrast with previous findings in Hungary (Tóth and Szabó 2011). The identification and separation of the *Cx. pipiens pipiens* and *Cx. pipiens molestus* forms were attempted by morphological analysis based on the key provided by Mohrig (1969). A 2011 collection of 11,728 mosquitoes assembled into 362 pools representing 24 different species and a 2012 collection of another 11,465 mosquitoes in 283 pools comprising 18 different species were tested by RT-PCR for WNV. A total of 23,095 of the mosquitoes were female; furthermore, 68 male *Cx. pipiens* and 30 male *A. maculipennis* were also captured during the winter period of 2011. All male mosquitoes tested negative for WNV. Positive RT-PCR results were obtained from two medium sized pools of the 2011 samples: A pool of female *O. annulipes* collected at Fényeslitke (location 1,

TABLE 1. NUMBERS OF MOSQUITOES TRAPPED AND TESTED FOR WEST NILE VIRUS, BY SPECIES

	Area I	Area II	Area III	Area IV	Area V	Total
<i>Aedes cinereus</i>	2206	202	44			2452
<i>Aedes rossicus</i>	356					356
<i>Aedes vexans</i>	8603	814	98			9515
<i>Anopheles algeriensis</i>	11					11
<i>Anopheles claviger</i>	14		6			20
<i>Anopheles hyrcanus</i>	876	11	183			1070
<i>Anopheles maculipennis</i>	113	2	4	166	16	301
<i>Anopheles plumbeus</i>	23		7			30
<i>Coquillettidia richiardii</i>	790	313	471			1574
<i>Culex hortensis</i>	1					1
<i>Culex martinii</i>	7					7
<i>Culex modestus</i>	323	255	116	2		696
<i>Culex pipiens</i>	87	2	2	178	101	370
<i>Culex territans</i>	5					5
<i>Culex torrentium</i>	8					8
<i>Culiseta alaskaensis</i>	1					1
<i>Culiseta annulata</i>				1	50	51
<i>Ochlerotatus annulipes</i>	395	28	68			491
<i>Ochlerotatus cantans</i>	29	29	221			279
<i>Ochlerotatus caspius</i>			20			20
<i>Ochlerotatus cataphylla</i>		1				1
<i>Ochlerotatus excrucians</i>		1				1
<i>Ochlerotatus flavescens</i>		25	39			64
<i>Ochlerotatus geniculatus</i>	9		1			10
<i>Ochlerotatus leucomelas</i>		2	3			5
<i>Ochlerotatus rusticus</i>	68					68
<i>Ochlerotatus spp.</i>				3		3
<i>Ochlerotatus sticticus</i>	5656	38	55			5749
<i>Uranotaenia unguiculata</i>	34					34

N48°18'44,75", E22°04'07,64") in June and a pool of female *C. richiardii* collected in Debrecen, Fancsika-tó (location 11, N47°30'36,37", E21°44'27,18") in July. Furthermore a large-sized pool of female *Cx. pipiens* captured near Red-footed Falcon colonies in Kardoskút (location 18, N46°29'49,00", E20°42'13,98") in September, 2011, was found positive for WNV RNA (Table 2, Fig. 1). All mosquito samples from 2012 tested negative for WNV.

We calculated the minimal infection rate (MIR = the proportion of infected mosquitoes per 1000 mosquitoes) in 2011, when WNV was identified in mosquitoes in Hungary. It was 0.25 regarding all collected mosquitoes, and when calculating by mosquito species, we found that MIR was 2.03 in *O. annulipes*, 0.63 in *C. richiardii*, and 2.70 in *Cx. pipiens*. Taking into consideration the variable pool sizes, we also used the EpiTools epidemiological calculator program (<http://epitools.ausvet.com.au/content.php?page=PPVariablePoolSize>) for estimating prevalence with 95% confidence intervals (CI). The calculated prevalence values were as follows: 3×10^{-4} (lower 95% confidence level [CL] = 10^{-4} , upper 95% CL = 7×10^{-4}) regarding all collected mosquitoes, 2×10^{-3} (lower 95% CL = 10^{-4} , upper 95% CL = 9×10^{-3}) in *O. annulipes*, 7×10^{-4} (lower 95% CL = 0, upper 95% CL = 2.9×10^{-3}) in *C. richiardii*, and 2.9×10^{-3} (lower 95% CL = 2×10^{-4} , upper 95% CL = 1.26×10^{-2}) in *Cx. pipiens*.

Discussion

Recent WNV activity in Hungary has been monitored through passive surveillance for more than a decade, but the

very first reports on the occurrence of WNV in the country date back to 1976 (Molnár 1982). Molecular data have demonstrated the exclusive occurrence of lineage 2 WNVs in wild birds, horses, humans, or mosquitoes each year since their first detection in 2004 (Bakonyi et al. 2013). Given that lineage 1 WNV strains were detected only before 2004, this finding suggests that lineage 2 WNVs became much more prevalent or were possibly the only lineage circulating in this country over the past 10 years. On the basis of the highly similar genomic sequences obtained from all viruses isolated from wild birds, horses, and mosquitoes in Hungary and the surrounding areas, it seems likely that the virus is able to maintain a year-round transmission cycle with competent overwintering mosquitoes and avian hosts, with the occasional involvement of dead-end hosts.

However, a decreased detection rate of WNV was observed in animal hosts during examination of dead wild birds and horses over the past few years in Hungary. In 2010 WNV RNA was detected in seven wild bird carcasses and in a horse (unpublished data), and furthermore 19 clinically affected horses were found to be immunoglobulin M (IgM) positive. In 2011, five wild birds and one horse specimen tested positive for WNV with RT-PCR, and in addition 19 horse blood samples were positive for anti-WNV IgM antibodies, whereas in 2012 only two wild bird carcasses were found positive by PCR and four affected horses showed IgM positivity. The number of reported human WNF cases also varied during these years: 15 confirmed cases in 2010, four in 2011, and 12 in 2012 (ECDC Cumulative number of West Nile fever cases).

TABLE 2. MOSQUITO POOLS BY MOSQUITO SPECIES TRAPPED AND TESTED FOR WEST NILE VIRUS IN HUNGARY, 2011–2012

Species	No. trapping locations	No. pools (total)	2011; 2012 ^b			Month of trapping
			No. pools with the indicated no. mosquitoes per pool			
			1–3	4–19	>20	
<i>Aedes cinereus</i>	8; 6	52; 16	8; 5	8; 1	36; 10	5,6,7,8,9; 5,6,7,8
<i>Aedes rossicus</i>	1; 2	4; 10	1; 1	2; 3	1; 6	5,6,7,8; 5,6,7,8
<i>Aedes vexans</i>	9; 9	94; 133	7; 25	9; 3	78; 105	5,6,7,8,9; 5,6,7,8,9
<i>Anopheles algeriensis</i>	1; 0	1; 0	1; 0	0; 0	0; 0	5; -
<i>Anopheles atroparvus</i>	0; 2	0; 2	0; 1	0; 0	0; 1	-; 2
<i>Anopheles claviger</i>	1; 0	3; 0	2; 0	1; 0	0; 0	7,8,9; -
<i>Anopheles hyrcanus</i>	5; 7	20; 20	4; 11	0; 3	16; 6	5,7,8,9; 5,6,7,8
<i>Anopheles maculipennis</i>	7; 6	13; 6	2; 2	5; 3	6; 1	5,6,7,8,9; 1,2,7
<i>Anopheles messeae</i>	0; 1	0; 1	0; 1	0; 0	0; 0	-; 1
<i>Anopheles plumbeus</i>	1; 2	2; 3	1; 3	1; 0	0; 0	6,8; 7,8
<i>Coquillettidia richiardii</i>	9; 9	35; 43	7; 17	11 ^a ; 9	17; 17	6,7 ^a ,8,9; 6,7,8,9
<i>Culex martinii</i>	1; 0	2; 0	1; 0	1; 0	0; 0	6,8; -
<i>Culex modestus</i>	9; 7	20; 22	6; 12	9; 9	5; 1	6,7,8,9; 5,6,7,8,9
<i>Culex pipiens molestus</i>	1; 0	1; 0	1; 0	0; 0	0; 0	12; -
<i>Culex pipiens pipiens</i>	7; 3	19; 5	5; 4	9; 0	5 ^a ; 1	6,7,8, 9 ^a ,12; 1,2,8
<i>Culex territans</i>	1; 0	1; 0	0; 0	1; 0	0; 0	7; -
<i>Culex torrentium</i>	1; 0	1; 0	0; 0	1; 0	0; 0	8; -
<i>Culiseta alaskaensis</i>	0; 1	0; 1	0; 1	0; 0	0; 0	-; 5
<i>Culiseta annulata</i>	1; 3	1; 3	1; 2	0; 0	0; 1	9; 1,2
<i>Ochlerotatus annulipes</i>	4; 4	22; 7	5; 7	7 ^a ; 0	10; 0	5,6 ^a ,7,8; 5,6,7,8
<i>Ochlerotatus cantans</i>	5; 0	16; 0	7; 0	4; 0	5; 0	5,6,7; -
<i>Ochlerotatus caspius</i>	0; 1	0; 3	0; 3	0; 0	0; 0	-; 7,8,9
<i>Ochlerotatus excrucians</i>	0; 1	0; 1	0; 1	0; 0	0; 0	-; 6
<i>Ochlerotatus flavescens</i>	3; 1	5; 1	1; 1	4; 0	0; 0	5,6; 6
<i>Ochlerotatus geniculatus</i>	2; 0	2; 0	2; 0	0; 0	0; 0	8; -
<i>Ochlerotatus rusticus</i>	1; 0	1; 0	0; 0	1; 0	0; 0	6; -
<i>Ochlerotatus sticticus</i>	5; 3	44; 3	3; 2	5; 1	36; 0	5,6,7,8,9; 7,8
<i>Ochlerotatus spp.</i>	1; 0	1; 0	1; 0	0; 0	0; 0	9; -
<i>Uranotaenia unguiculata</i>	1; 0	2; 0	1; 0	0; 0	1; 0	7,8; -

^aPositive samples.^bNumbers in italics refer to 2012.

Given that passive surveillance is highly sensitive to the quality and quantity of samples received for testing, a decreasing number of total WNV-positive cases has to be evaluated with caution. One of the potential explanations for the high number of positive cases in 2010 and the subsequent decrease in 2011 and 2012 may be found in the weather conditions and the correlated density of mosquitoes. The year 2010 was extremely rainy with 959 mm of rain, whereas the average yearly precipitation in Hungary is between 500 mm and 750 mm. 2010 was the rainiest year since 1901; the second in this order was the year 2005 with 748 mm. In contrast, the two following years were extremely dry; moreover, 2011 broke the record for the driest year since 1901, with 407.4 mm (Hungarian Meteorological Service; www.met.hu), although the early floods of that same year could have provided ideal conditions for mosquito populations (Hubalek 2008).

Virus introduction, amplification, and expansion are probably the most important keystone events and sequential stages of WNV epidemiology in the European context. The exact prerequisites needed for the successful completion of these events are not entirely known. It could be assumed that WNV-infected individuals can be regularly found among the migratory birds reaching continental Europe on their spring

migration. However, whether the virus will encounter suitable populations of mosquito vectors necessary for the initiation of local circulation and whether these vector and host species would be abundant enough for the amplification and subsequent epidemic spread of WNV is probably primarily influenced by climatic conditions and by an additional range of environmental factors. Accumulating evidence suggests, that the current dominance of lineage 2 WNV circulation in Hungary and Greece and the simultaneous presence of lineages 1 and 2 WNV in Italy is probably the result of the combination of largely random introduction events and the subsequent, more predictable expansion of the established virus strains.

Cx. pipiens and *C. richiardii* are considered the principal mosquito vectors of WNV in Europe (Higgs et al. 2004, Lvov et al. 2004, Hayes et al. 2005, Reiter 2010). *Cx. pipiens* is essentially an ornithophilic mosquito species; however, it has been demonstrated that a proportion of their blood meal may be taken from mammals, including humans (Zimmerman et al. 1985, Gómez-Díaz and Figuerola 2010). *Cx. pipiens* is common in urban and suburban areas, but it is also widespread in rural habitats. Overwintering *Culex* mosquitoes are able to survive the winter under continental climate and amplify the virus in spring, when increasing day length and

warming temperatures terminate their diapause and females seek a blood meal (Spielman and Wong 1973, Cornel et al. 1993, Nasci et al. 2001, Anderson and Main 2006, Farajollahi et al. 2011). Vertical transmission of the virus by mosquitoes had been demonstrated in the laboratory (Baqar et al. 1993, Goddard et al. 2003) and under field conditions (Miller et al. 2000) as well. A 168-day-old vertically infected female *Cx. pipiens* was found competent to transmit WNV horizontally to a hamster, which died of WNV infection 8 days after being bitten (Anderson and Main 2006). These results demonstrate that a vertically infected *Cx. pipiens* that entered diapause in late autumn is able to initiate infection the following spring. Infected females contribute to avian and human infections by horizontal transmission of the virus during their first and subsequent blood feedings (Anderson and Main 2006). *Cx. pipiens* is a widespread mosquito in Hungary (Kenyeres and Tóth 2008), as many types of breeding sites are suitable for this species. *C. richiardii* is widespread throughout Europe; its larval stage is able to overwinter, even in ice-bound waters. Adults are active from May to September with the highest density in July and August. They can reach very high densities in the surroundings of freshwater bodies and attack humans aggressively. They feed mainly on mammals and occasionally on birds, and they prefer to feed on humans mainly indoors (Service 1971). In Hungary *C. richiardii* is a common species near wetlands; it prefers reeds, because the larvae breed only in water rich in organic matter and suspended micro-organisms (Schaffner et al. 2001). In Central Europe *C. richiardii* is univoltine, but it produces two to three generations in southern countries. *O. annulipes* is also widespread throughout Europe, and it overwinters in the egg stage. Adults are active from April to September and they usually breed in open meadow pools and at deciduous forests. Females are daytime biters with a crepuscular activity, feeding principally on mammalian blood. They are known to be bridge vectors of WNV between birds and mammals, including horses (Kulasekera et al. 2001, Becker et al. 2003). In addition, *O. annulipes* shows a strong preference for human blood meal (Zamburlini 1996). In Hungary *O. annulipes* lives mainly in the lowlands (Tóth 2004) and breeds especially in forests influenced by snow-floods.

Several investigations had been performed in different European countries with the purpose to measure the role of various mosquito species in the maintenance of the WNV transmission cycle (Engler et al. 2013). The MIR refers to the proportion of infected mosquitoes per 1000 mosquitoes. Earlier studies established that the WNV MIR value in *Cx. pipiens* mosquitoes was 0.19 in Romania in 1997 (Savage et al. 1999), 0.08 in the Czech Republic between 1997 and 1999 (Hubálek 2000), 0.09 in Belarus between 1985 and 1987 (Samoilova et al. 2003), 0.23 in Portugal regarding all collected mosquitoes and 1.93 in *Cx. pipiens* between 2001 and 2004 (Almeida et al. 2008), 0.35 regarding the total number of mosquitoes, and 0.26 in *Cx. pipiens* and 0.49 in *C. richiardii* in Russia between 2001 and 2002 (Lvov et al. 2004). The estimated prevalence of WNV infection in mosquitoes in Hungary was comparable to that reported by other studies from Europe.

In the current study, we analyzed mosquito material from several, initially independent sources. In 2011, the first WNV-positive mosquito was found in June; however, we could not detect any positive pool in 2012 despite the occurrence of human cases. Nevertheless, the results of the survey have

corroborated the findings of the host case surveillance and have provided baseline data for further targeted vector surveillance. We consider this kind of *ad hoc* sample and data collation effort rather useful for bridging knowledge gaps in the absence of a comprehensive vector surveillance system. An efficient, well-organized mosquito surveillance program based on regular sampling would be required for the early detection of emerging WNV activity in mosquito populations. Monitoring of mosquitoes could provide early information about the appearance of new mosquito-borne viruses in an unaffected region and about variations in the composition of circulating virus strains in endemic areas. Also it may indicate the rising level of virus circulation prior to the detection of increased seroconversion or symptomatic disease manifestations in animals or humans.

Conclusion

The present study clearly indicates that surveillance for WNV needs to rest on multiple pillars. Dead bird testing, serology, and direct virus detection based surveillance of WNV in domestic animals (horses) and humans have been practiced previously in our country, but mosquito-based surveillance may prove equally important for the early detection of WNV circulation, allowing some time for the establishment of preventive procedures, e.g., horse vaccination or human protection against mosquito bites. Nevertheless, mosquito surveillance alone is not sufficient to adequately evaluate the veterinary and public health risk of WNV infection. However, a reasonably well-organized mosquito surveillance system would provide valuable information on the current epidemiological status of an area. Furthermore, it could be a useful tool in countries potentially threatened by WNV emergence.

Acknowledgments

Mihály Tóth's involvement in this research was supported by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP 4.2.4. A/2-11-1-2012-0001 National Excellence Program. K. Erdélyi was supported by the Bolyai János Research Scholarship of the Hungarian Academy of Sciences. The study was partially supported by the TÁMOP-4.2.2.B-10/1 and TÁMOP-4.2.1.B-11/2/KMR-2011-0003 projects, and by the European Union grant FP7-261504 EDENext (www.edenext.eu) and is catalogued by the EDENext Steering Committee as EDENext 189. The contents of this paper are the sole responsibility of the authors and do not necessarily reflect the views of the European Commission.

Author Disclosure Statement

No competing financial interests exist.

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