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West Nile virus 'circulation' in Vojvodina, Serbia: Mosquito, bird, horse and human surveillance

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ABSTRACT

Efforts to detect West Nile virus (WNV) in the Vojvodina province, northern Serbia, commenced with human and mosquito surveillance in 2005, followed by horse (2009) and wild bird (2012) surveillance. The knowledge obtained regarding WNV circulation, combined with the need for timely detection of virus activity and risk assessment resulted in the implementation of a national surveillance programme integrating mosquito, horse and bird surveillance in 2014. From 2013, the system showed highly satisfactory results in terms of area specificity (the capacity to indicate the spatial distribution of the risk for human cases of West Nile neuroinvasive disease - WNND) and sensitivity to detect virus circulation even at the enzootic level. A small number (n = 50) of *Culex pipiens* (*pipiens* and *molestus* biotypes, and their hybrids) females analysed per trap/night, combined with a high number of specimens in the sample, provided variable results in the early detection capacity at different administrative levels (NUTS2 *versus* NUTS3). The clustering of infected mosquitoes, horses, birds and human cases of WNND in 2014–2015 was highly significant, following the south-west to north-east direction in Vojvodina (NUTS2 administrative level). Human WNND cases grouped closest with infected mosquitoes in 2014, and with wild birds/mosquitoes in 2015. In 2014, sentinel horses showed better spatial correspondence with human WNND cases than sentinel chickens. Strong correlations were observed between the vector index values and the incidence of human WNND cases recorded at the NUTS2 and NUTS3 levels. From 2010, West Nile virus was detected in mosquitoes sampled at 43 different trap stations across Vojvodina. At 14 stations (32.56%), WNV was detected in two different (consecutive or alternate) years, at 2 stations in 3 different years, and in 1 station during 5 different years. Based on these results, integrated surveillance will be progressively improved to allow evidence-based adoption of preventive public health and mosquito control measures.

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1. Introduction

West Nile virus (WNV), family *Flaviviridae*, is the most widespread arbovirus in the world. It started to cause growing concerns in Europe after the largest outbreak in Romania in 1996, which was connected to many cases of neuroinvasive disease (WNND) in humans [1,2].

The virus may disappear or remain undetected for long periods, but different environmental "drivers", such as an increase in temperature, may enhance virus circulation and affect humans and equids [3]. In Europe, the main vector is *Culex pipiens* (including the *pipiens* and *molestus* biotypes, and their hybrids) [1,4,5], but *Cx. perexigus* and *Cx. modestus* may act as a bridge between birds and humans/horses in specific areas [6]. The virus may overwinter in infected female mosquitoes, as well as in residential birds, and could circulate locally without re-introduction via migrating birds [7–9].

The largest WNV outbreak in Europe, with more than 390 confirmed cases, was reported in Romania in 1996 [1]. Serbia experienced the second largest outbreak, with 200 confirmed human cases in 2013 [10]. The largest numbers of human cases, as well as outbreaks of various magnitudes, have been reported in the Vojvodina province of northern Serbia since 2012 (9 in 2012, 85 in 2013, 27 in 2014 and 10 in 2015) [11]. According to the nomenclature of territorial units for statistics (NUTS), Vojvodina corresponds to the NUTS2 administrative level [12].

Little is known about the history of WNV circulation in Serbia. The first serological investigation was conducted in 1972, and antibodies against WNV were found in 2.6%–4.7% of human serum samples [13]. After more than a 30-year gap, the authors of this paper resumed serological investigation, first of human sera. ELISA IgG testing revealed that the seroprevalence of WNV was 6.67% in human sera from 45 patients who had been hospitalized for encephalitis or meningoencephalitis between 2001 and 2005. The seroprevalence was 3.69% in 406 samples taken from healthy people. The average seroprevalence of WNV in samples taken from 2001 to 2009 was 3.99% (18 of 451). A total of 337 individuals tested in 2009 were exposed to at least one mosquito exposure-related risk factor. Within this group, 5.04% were seropositive for WNV. Most of the probably infected people (those with IgG in blood sera) did not have screens on the windows and doors of their houses, while only 0.88% of those using window screens were seropositive for WNV [5]. During the same period, 56,757 mosquito specimens, sampled at migratory and domestic bird reservoirs, were pooled into 841 samples and all tested negative for WNV RNA. A serological analysis by ELISA based on WNV recombinant envelope E (rE) protein and PRNT showed, for the first time in Serbia, that 12% of 349 horses from the northern part of country sampled from 2009 to 2010 presented with specific neutralizing WNV antibodies [14]. Due to the absence of routine diagnosis and the limited resources of hospitals in Serbia, human cases of meningoencephalitis of unknown origin that should have been evaluated by a plaque reduction neutralization test and/or RT-PCR tests for WNV were not tested until 2012. In addition, regular sentinel chicken, horse or mosquito surveillance did not exist. Consequently, the approach used to search for the virus in Serbia had been focused on IgG-positive humans to find infected mosquitoes, i.e., to provide evidence of circulation and raise the public awareness of the risk. In accord with the negative results, the lack of the resources and resultant planning, the sampling of mosquitoes in 2010 and 2011 was performed at/around places where humans or equids that were IgG-positive or infected with WNV were recorded in the previous season. The plan implemented had generated initial results in 2010 when WNV linage 2 RNA was detected in three pools of *Cx. pipiens* [5]. In August 2012, a clinical outbreak of WNV infection in humans was reported for the first time in Serbia [15,16]. In addition, during the same year, viral RNA was detected for the first time in nine wild birds. All of the isolates were classified by phylogenetic analysis as lineage 2 WNV strains and were closely related to the strains responsible for recent outbreaks in Greece, Italy and Hungary [17].

The prevention and control of WNV outbreaks is complex and requires the implementation of a comprehensive surveillance system [18,19]. Environmental surveillance based on mosquito and/or bird collections and subsequent screening for the pathogen has been shown to perform well in detecting the virus circulation well before the occurrence of human cases, and also enables estimations of the magnitude of human WNV outbreaks, with a possibility for the identification of affected areas [18,20]. During the first period, from 2005 to 2013, WNV surveillance activities in Vojvodina were performed as part of ongoing research projects coordinated by the authors of this paper. In 2014, a specific and integrated surveillance system targeting mosquitoes, wild and sentinel birds as well as horses, was set up by the National Veterinary Directorate in Vojvodina province, northern Serbia. The main goals of this nationwide WNV surveillance have been to provide warnings of WNV circulation and evidence-based tools for controlling the spread of WNV infections in humans.

In this paper, the development of the surveillance system in the five years following the first detection (2010) of WNV RNA in *Cx. pipiens* mosquitoes in Serbia is presented. We also present a comparison between the results obtained from mosquito, bird, horse and human populations from 2014 to 2015 and describe their spatial clustering in Vojvodina province. In addition, the incidence of human WNND cases was correlated to the vector index values for *Cx. pipiens* for different sizes of territorial/administrative levels.

2. Material and methods

Vojvodina has a total surface area of 21,506 km^2 with a population of 1.93 million. The climatic and ecological conditions for *Cx. pipiens* (including biotypes *pipiens* and *molestus*, and their hybrids) development (e.g., availability of breeding sites, bird species populations and environmental parameters) are very similar all around the province and are considered appropriate for WNV circulation.

2.1. Surveillance of mosquitoes

Following the detection of the WNV infection in mosquitoes in the Vojvodina province in 2010 [5], a small-scale surveillance network was designed and operated during the late summer period (August–September) (Table 1). In the 2011-13 seasons, mosquito collections were conducted at places where humans or equids that were IgG-positive or infected with WNV had been recorded in the previous season. Surveillance in 2013 covered all of the districts of Vojvodina with human WNND cases. Traps baited with dry ice and without light (NS2 type) were operated from the afternoon until the morning of the next day (one trap night), some were set at fixed positions and others were irregularly placed, with weekly to biweekly periodicity.

From the 2014 season, the mosquito collections were standardized and traps were fixed to geo-referenced positions with biweekly periodicity (June–September) within high-risk (estimated according to number of human cases in the previous season) districts (NUTS3 level) of the province (NUTS2 level). In 2014, all districts except the North Banat district were considered to be high-risk. North Banat was sampled at a three-weekly or monthly periodicity (five times between June–September). In 2015, biweekly sampling was performed in all districts. The network was initially designed to cover the area of all districts and as many

Table 1
The surveillance activities in the Vojvodina province, Serbia, from 2009 to 2015 (data on the NUTS2 - provincial level).

Year	Trap nights	Period of mosquito sampling	Date of the first WNV positive mosquito pool	Sentinel chicken sampled/ positive	Date of the first WNV positive chicken	Wild birds collected/ positive (n)	Date of the first WNV positive bird	Horses sampled/ positive (n)	Date of the first WNV positive horse	Date of the first human WNND case	Human WNND cases (n)	Incidence WNND (cases/ 100,000)
2009	np	na	na	np	na	np	na	120/10	7.04	na	na	na
2010	38	Sep–Oct	02.09	np	na	np	na	229/32	01.03	na	na	na
2011	32	Sep	06.09	np	na	np	na	np	na	na	na	na
2012	39	Aug–Sep	02.08	np	na	82/9	10.06*	130/64	na**	15.07	9	0.47
2013	86	July–Sep	17.07	np	na	np	na	96/45	na**	12.07	85	4.40
2014	414	Jun–Sep	16.07	566/10	12.06	111/2	29.07	89/13	15.07	09.07	24	1.24
2015	492	Jun–Sep	13.06	np	na	73/7	14.08	326/11	28.09	10.08	10	0.52

np - not performed; na - not applicable; * sampled January–September; ** sampled November–December.

municipalities as possible using a grid of local roads (Fig. 1). The specific location of the trap in each municipality was chosen by experienced entomologists to stabilize *Cx. pipiens* collections.

Mosquitoes were collected, put on dry ice and maintained under "cold chain" conditions until the detection of viral RNA. They were identified to the species level [21], counted and pooled according to date, location and species, with a maximum number of 200 specimens per pool that were submitted for further analysis. Pooled mosquitoes were stored in freezers at −80 °C. Only one sub-pool of a maximum of 50 randomly chosen mosquitoes/trap/night/species was analysed. Before 2014, all mosquito species were pooled and subjected to virus detection, while in 2014 and 2015, pools were prepared from *Cx. pipiens* specimens only. The remaining mosquitoes were identified to the species level to allow calculation of vector index. In total, 410,469 mosquito females belonging to 17 species were sampled (Table 2) and 1303 pools were analysed for the presence of WNV RNA. Damaged, unidentifiable specimens and males are not shown in the table.

2.2. Surveillance of birds

The West Nile virus national surveillance plans in 2014 and 2015 envisaged the screening of live-captured and dead/hunted wild birds (Passeriformes, Accipitriformes). Tissues from dead birds and tracheal/pharyngeal swabs from live-trapped birds were tested individually for the presence of WNV. All dead birds and up to 100 tracheal swabs of trapped ones were tested during the whole year in the districts with high WNV risk. In the low-risk districts, up to 50 dead/shot wild birds collected from May to October were tested.

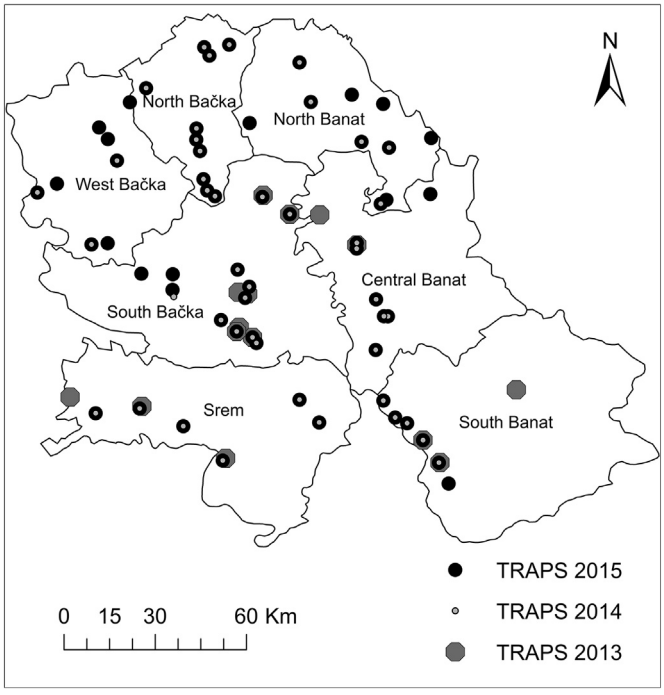

Fig. 1. The allocation of mosquito collection sites in the target area for West Nile virus entomological surveillance in Vojvodina, Serbia, from 2013 to 2015.

In 2014, sentinel chickens were also used (blood samples from 5 backyard chickens in 10 settlements per district were taken six times from May to October). On many occasions, it was difficult to confirm the sentinel status of the sampled chickens. Because of this uncertainty, the method was abandoned in 2015. In this paper, chickens with doubtful sentinel status were not considered. From dead birds, blood exudates were collected from the heart or from the pleural/abdominal cavity. A pool of selected tissues (brain, kidney, liver, lung and spleen) was prepared for each dead bird. Blood serum samples were examined for the presence of anti-WNV antibodies. Pools of tissue samples from birds were homogenised [22,23] and examined for the presence of WNV RNA. The detection of infective virus was performed on Vero cell cultures using standard procedures [24]. Table 1 shows the development and structure of the surveillance system, along with published data from 2012 [17].

2.3. Laboratory analyses of mosquitoes and birds

For WNV RNA detection in mosquito pools and bird samples, an in-house TaqMan-based one-step reverse transcription real-time PCR (RT-qPCR) that amplifies both lineage 1 and 2 strains, with primers and TaqMan probe described by Linke et al. [25], was used. Viral RNA was extracted using the commercial ISOLATE II RNA Mini Kit (Bioline, The Netherlands) according to the manufacturer's instructions. One-step RT-qPCR was conducted using the commercially available RNA UltraSense™ One-Step qRT-PCR System (Life Technologies Corporation) with primers (forward WNproC-F10: 5′-CCTGTGTGAGCTGACAAACTTAGT-3′ and reverse WNproC-R153: 5′-GCGTTTTAGCATATTGACAGCC-3′) and a probe (WNproC-probe 5′-FAMCCTGGTTTCTTAGACATCGAGATCT-TAMRA-3′) that targeted the nucleocapsid protein C gene regions of both WNV lineages 1 and 2 [25]. Each reaction contained 15 μl of reaction mix containing 1 X RNA UltraSense reaction mix, 20 μM of each primer, 10 μM of WNproC probe, 1 X ROX reference dye and 1 μl of RNA UltraSense enzyme mix. A 5 μl sample of nucleic acid extract was added to make a final reaction volume of 20 μl. The thermocycling conditions were 15 min at 50 °C, 2 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and 50 s at 60 °C.

2.4. Surveillance of horses

Serum samples from 100 healthy horses/district, with no clinical

Table 2
The mosquito species sampled, number of specimens collected and pools tested in Vojvodina, Serbia, from 2010 to 2015.

Species	Year												Total		
	2010		2011		2012		2013		2014		2015				
	ns	npt*	*ns*	npt*	*ns*	npt*	*ns*	npt*	*ns*	npt**	*ns*	npt*	*ns*	npt*	Positive
Anopheles hyrcanus	–	–	–	–	*4*	3	*24*	5	*346*	0	*175*	0	*549*	8	0
Anopheles maculipennis s.l.	*6*	2	–	–	*5*	4	*146*	24	*754*	0	*202*	0	*1113*	30	0
Anopheles plumbeus	–	–	–	–	–	–	*1*	1	–	–	*8*	0	*9*	1	0
Aedes cinereus	*1*	1	–	–	–	–	*1*	1	*5*	0	*3*	0	*10*	2	0
Aedes rossicus	–	–	–	–	–	–	*1*	1	*5*	0	*451*	0	*6*	1	0
Aedes vexans	*11*	7	–	–	*107*	13	*319*	26	*26142*	0	*2*	0	*26581*	46	2
Aedes annulipes	–	–	–	–	–	–	*2*	1	*20*	0	–	–	*22*	1	0
Aedes cantans	–	–	–	–	–	–	–	–	*492*	0	*115*	0	*607*	0	0
Aedes caspius	*11*	6	–	–	*78*	10	*51*	9	*1759*	0	–	–	*1899*	25	0
Aedes geniculatus	–	–	–	–	–	–	*1*	2	*10*	0	–	–	*11*	2	0
Aedes sticticus	–	–	–	–	–	–	*3*	2	*7122*	0	*23*	0	*7148*	2	0
Aedes albopictus	–	–	–	–	–	–	*5*	2	–	–	–	–	*5*	2	0
Culex pipiens	*1488*	39	*510*	40	*6520*	154	*20751*	179	*251538*	375	*81648*	337	*362455*	1124	95
Culex modestus	–	–	*2*	2	–	–	*2*	1	*24*	0	*181*	0	*209*	3	0
Culiseta annulata	*3*	2	*1*	1	*9*	6	*249*	26	*1114*	0	*201*	0	*1577*	35	2
Coquillettidia richiardii	–	–	*1*	1	*5*	2	*55*	17	*5355*	0	*2851*	0	*8267*	20	0
Uranotaenia unguiculata	–	–	–	–	*1*	1	–	–	–	–	–	–	*1*	1	0
Total**	*1520*	57/3	*514*	44/3	*6729*	193/21	*21611*	297/26	*294686*	375/23	*85860*	337/20	*410469*	1303	99

ns – number of specimens sampled; npt – number of pools tested; * number of mosquitoes per pool 1–50; ** number of pools/number of positive pools for all species.

sign of disease, of different ages and breeds, were tested for the presence of anti-WNV IgG antibodies by commercial blocking ELISA kits (INGEZIM WEST NILE COMPAC, Ingenasa, Spain) following the manufacturer's instructions. Testing was performed during the period from April–May 2014 to mark sentinels for the subsequent surveillance season. All negative horses were used as sentinels (Table 1). Up to 50 horses per high-risk district and 30 sentinel horses per low-risk district were sampled three times during the season (in June, July and August) from at least 3 sample sites per district. In 2015, due to the high number of seropositive horses, the surveillance in horses was performed by detecting IgM antibodies (ID Screen West Nile IgM Capture, IDvet, France). Horses were sampled at at least 7 different sites per district. The samplings and biomolecular analysis were performed by veterinarians from the regional station network and sent to the National Reference Centre (Veterinary Institute Kraljevo) for confirmation, sequencing and determination of the lineage. Blood samples were taken from the jugular vein and transported to the laboratory within a few hours. Table 1 shows the development and structure of the surveillance system, as well as published data from 2009 to 2012 [14,26]. All WNV-positive samples in 2014 and 2015 were sent to the National Reference Centre (Veterinary Institute Kraljevo) for confirmation, sequencing and determination of the lineage.

2.5. Surveillance of humans

During the study period, the surveillance of WNV in the human population was conducted in May–November each year. The criteria for a clinical and laboratory diagnosis and for case classification were set according to CDC (Atlanta, GA, USA) recommendations [27]. Patients who met the clinical criteria for neuroinvasive arboviral disease (fever $\geq$ 38 °C, and at least one sign of central or peripheral neurological dysfunction such as meningitis, encephalitis, acute flaccid paralyses and the absence of a more likely clinical explanation) or for non-neuroinvasive arboviral disease (fever $\geq$ 38 °C, absence of neuroinvasive disease, and the absence of a more likely clinical explanation) were suspected of being infected with WNV and were subjected to laboratory testing.

Serum samples from non-neuroinvasive or serum and cerebrospinal fluid (CSF) samples from neuroinvasive cases were collected and tested for the presence of specific anti-WNV IgM and IgG antibodies using commercially available ELISA kits ("Euroimnune", Germany) on a Euroimmune Analyzer I ("Euroimnune", Germany). Due to the possible serological cross-reactivity between different flaviviruses, serum samples were also analysed for the presence of IgM and IgG antibodies against dengue virus, Usutu virus, tick-borne encephalitis virus, and yellow-fever virus. If the time period from symptom onset to sampling was no more than 7 days, samples were also analysed for the presence of WNV RNA by real-time RT-PCR. Nucleic acid extraction was performed with a QIAamp Viral RNA Mini Kit ("Qiagen", Germany) on a QIAcube ("Qiagen", Germany). Real-time RT-PCR testing was performed using WNV Real-TM ("Sacace", Italy) in an Applied Biosystems 7500 thermocycler. All analyses were performed following the manufacturers' instructions. The suspected cases were classified as laboratory-confirmed cases of WNV disease if the results of the virological analysis showed the presence of WNV RNA in serum or CSF samples or the presence of virus-specific IgM antibodies in the CSF and negative results for IgM antibodies specific for other arboviruses. The cases were considered to be probable cases of WNV infection if only specific IgM antibodies against WNV were found in a serum sample.

Although WNV infection is not a reportable disease in Serbia, according to national legislation, every confirmed neuroinvasive case was reported to the Institute of Public Health of Serbia "Milan Jovanovic Batut". Biological samples were transported to the National Laboratory for Viral Haemorrhagic Fevers and ARBO Viruses within the Institute of Virology, Vaccines and Sera "Torlak".

2.6. Statistical analysis

The spatial clustering of human, mosquito, horse and bird WNV cases for 2014 and 2015 was evaluated by the Average Nearest Neighbor statistics in ArcMap (ESRI 2010) [28]. A Z score below −2.58 and a p-value under 0.01 for both years implies a less than 1% likelihood that the clustered pattern could be a result of random chance. For an Average Nearest Neighbor ratio (ANN) below 1, the pattern is considered to be clustered. For ANN>1, the features are considered to be dispersed. The Directional Distribution (DD) tool (ESRI 2010) was used to create a standard deviation ellipse to summarize the central tendency, dispersion, and directional trends of the data. One standard deviation ellipse covers

approximately 68 percent of the features from the dataset. Furthermore, a statistical summary of the inter-point dependence and clustering was made by the Kest function using the "spatstat" R package [29], which calculates Ripley's reduced second moment function from a point pattern. The spatial similarity of the distribution pattern for human-mosquito, human-bird and human-horse cases in 2014 and 2015 was calculated by the Point Distance Analyst (PD) tool (ESRI 2010). The averaged PD value is smaller for patterns with a high degree of similarity (0 for identical patterns). The averages of three different (human-mosquito; human-bird; human-horse) sets of PD values were compared using a one-way ANOVA and the Newman-Keuls test (STATISTICA version 12, data analysis software system, Dell Inc. 2015).

The vector index (VI) was calculated corresponding to the traps running on a biweekly/monthly basis in 2013–2015 using the formula VI = ΣNi Pi (where N is the average number of *Cx. pipiens* collected per trap/night and P is the Maximal Likelihood Estimation (MLE) of infection, estimated using the PooledInfRate 4.0 software) [30]. As traps were activated mainly with bimonthly periodicity, a series of VI values were obtained during each season; "start VI" is the first VI value in the season; "max VI" is the maximum value reached during the season and "avg VI" was the mean seasonal value in a province/group of districts. A linear regression analysis was used to estimate the correlation between VI and the seasonal incidence of human WNND cases, using the STATISTICA version 12 data analysis software system (Dell Inc. 2015). The incidence of WNND cases was transformed to log(1 + case incidence) to normalise the data and control for variance. The correlation was estimated at the province level (NUTS2) and the two groups of districts (NUTS3) categorised according to the WNND case clustering.

3. Results

West Nile virus was detected in 99 of 1303 pools of mosquitoes sampled from 2010 to 2015 (Table 2). In 2012, two *Aedes vexans* pools and two *Culiseta annulata* were found to be positive. The rest of the 95 positive pools were composed of *Cx. pipiens* females.

West Nile virus activity was recorded in the Vojvodina province in 2009 (horses - E), 2010 (mosquitoes – M), 2011 (M), 2012 (M, E, wild birds – WB and humans – H), 2013 (M, E and H), 2014 (M, E, WB, H and sentinel chicken-SC) and 2015 (M, E, WB and H). In 2010 [5] and the following years, only the WNV lineage 2 strain was found. At the province level (NUTS2), wild birds were the first to signal the onset of human cases in 2012, when the first positive bird was detected one month before the first human WNND case (Table 1). In 2013, the first human case was recorded five days before detection of the first positive mosquito pool, but in 2014, a sentinel chicken was the first to indicate the virus circulation, four weeks before WNND cases were detected. In 2015, mosquitoes were first found to be infected, indicating virus circulation, two months prior to the first human case. From 2010, WNV was detected in mosquitoes sampled at 43 different trap stations across Vojvodina. At 14 stations (32.56%), WNV was revealed in two different (consecutive or alternate) years, WNV was detected at 2 stations in 3 years, and in 1 station during different 5 years (Table 1, Appendix).

The collected data from 2013 to 2015 allowed for a more precise analysis at the district level (Table 3). This was not possible in 2010/2011 or in 2012 because the surveillance system was confined to relatively small areas constrained by a lack of project funding and was not adequately standardized. In 2010 and 2011, we did not record any WNND cases, but WNV circulation was detected in September in one district (South Bačka). From 2013 to 2015, mosquitoes indicated the virus circulation on 6 of 10 occasions (indicating the capacity for early detection) and when it happened, it was 1–9 weeks before the detection of human cases. The sensitivity of surveillance also improved in 2014 and 2015 when enzootic WNV circulation was confirmed by detection of virus in mosquitoes in both years in Central Banat, North Bačka, North Banat (only 2014) and West Bačka districts without any human cases being recorded (Table 3).

Ripley's K-function and the Average Nearest Neighbor analysis proved that there was significant clustering of the mosquito, bird, horse and human cases in both 2014 (ANN = 0.463375, z-score: −11.705051, p-value: 0.000000) and 2015 (ANN = 0.671317, z-score: −4.356416, p-value: 0.000013). Furthermore, a Directional Distribution Analysis showed that the cases were distributed across the south-west to north-east transect of Vojvodina in both years (NUTS2 level – Fig. 2a, b). A similarity analysis (PD) revealed high spatial matching between the distribution pattern of human WNND cases and mosquitoes in 2014 (M = 0.8301, E = 0.902, SC = 0.956) and wild birds in 2015 (WB = 0.860, M = 0.868, E = 0.996).

Both the VI and human incidence rates were highest in 2013 and lowest in 2015. The maximum VI value of 3.33 was recorded in 2013 for the western group of districts (North Bačka, South Bačka, Srem and West Bačka). The VI value had been decreasing since 2013, and in 2014 and 2015, the value was higher for the eastern group of districts (North Banat, Central Banat and South Banat) (Table 4).

The correlation between the VI values and incidence of WNND cases at the province level (NUTS2) was positive, very high and significant with $r = 0.999$ ($p < 0.05$) for the maximum VI (highest seasonal value); positive, high and not significant with $r = 0.845$ ($p > 0.05$) for the average VI (average value for July–September). Datasets were also downscaled, and the values were calculated for two groups of districts (NUTS3) arranged according to the east – west human WNND cases grouping (Table 4). A positive, high, and significant correlation was found at the NUTS3 level for the maximum VI with $r = 0.871$ ($p < 0.05$); a positive, very high, and significant correlation was found for the average VI with $r = 0.945$ ($p < 0.05$) (Fig. 3); and a positive, low and not significant correlation with $r = 0.17$ ($p > 0.05$) was found for the starting VI (first seasonal VI).

4. Discussion

The WNV surveillance system in Vojvodina has been under development since 2009, and the last two years (2014 and 2015) has shown encouraging results, in terms of the sensitivity (capacity to detect WNV circulation at the enzootic level) and area specificity (capacity to indicate the spatial distribution of the risk for human WNND cases). In the 2014 season, WNV activity was detected in four out of seven districts (Central Banat, North Bačka, North Banat and West Bačka) at a very low level and where no human cases were registered. The three remaining districts had positive mosquitoes matched with human cases. A similar situation was noted in the 2015 season for the Central Banat, North Bačka and West Bačka districts. Wild birds were not sampled systematically; hence, only a small number of samples was analysed annually. However, the testing of wild birds signalled the circulation of WNV one month before the first human case in 2012. In 2014, sentinel chickens signalled WNV circulation 34 days before it was detected in mosquitoes, but the sentinel chickens were abandoned in 2015 because of difficulties in determining the status of the chickens (sentinel or not) during numerous interviews with the owners.

The standardized entomological surveillance (2013–2015) provided data that allowed for comparative analyses performed at the province (NUTS2) and district (NUTS3) levels. The results obtained at the district level allowed for a more precise estimation of the

Table 3
The seasonal onset of the first positive mosquito pool and the first human case of West Nile neuroinvasive disease (WNND) at the district level (NUTS3) in Vojvodina, Serbia, from 2013 to 2015.

District	Year								
	2013			2014			2015		
	Date of the first positive mosquito pool	Date of the first human WNND case	Lag time (days) between human and mosquito	Date of the first positive mosquito pool	Date of the first human WNND case	Lag time (days) between human and mosquito	Date of the first positive mosquito pool	Date of the first human WNND case	Lag time (days) between human and mosquito
Central Banat	23.07	08.08	−16	16.07	nd	nc	11.08	nd	nc
North Bačka	ns	22.07	nc	16.07	nd	nc	11.08	nd	nc
North Banat	ns	15.08	nc	11.09	nd	nc	nd	nd	nc
South Bačka	23.07	12.07	11	16.07	09.07	7	13.06	17.08	−65
South Banat	17.07	31.07	−14	30.07	11.08	−12	22.08	10.08	12
Srem	30.07	22.07	8	16.07	26.07	−10	07.07	19.08	−43
West Bačka	ns	09.08	nc	11.09	nd	nc	29.08	nd	nc

ns: not sampled; nd: no positive mosquito pool or no WNND case detected; nc: not calculated because mosquitoes were not sampled or no positive mosquito pool and/or WNND case were detected.

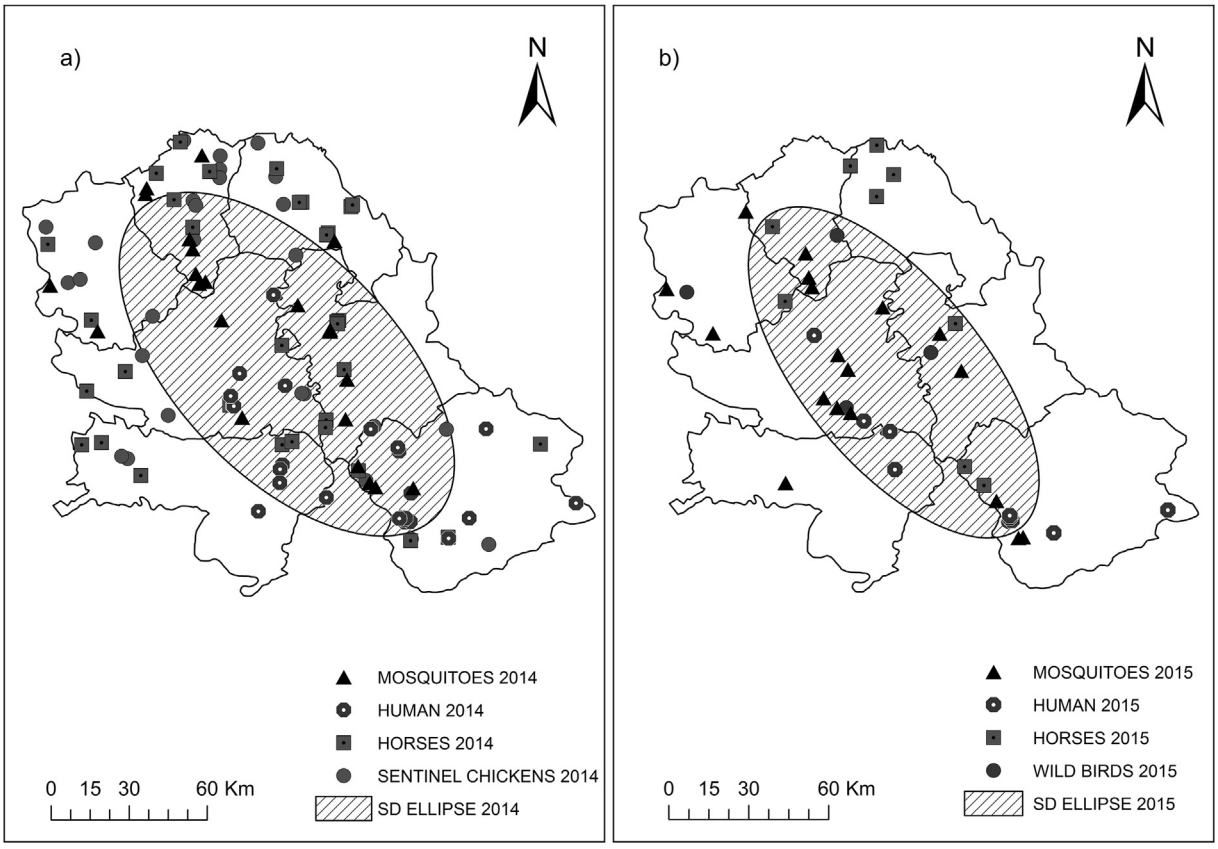

Fig. 2. The clustering of mosquito, bird, horse and human cases in 2014 (a) and 2015 (b) in Vojvodina, Serbia, from 2014 to 2015.

system's sensitivity and capacity for early detection.

Even though the sampling of horses had been performed in a relatively small number of stables at already fixed places, horse surveillance revealed a higher degree of spatial similarity to human cases than sentinel chickens in 2014. However, an additional disadvantage of horse surveillance was the need for laborious testing during the winter/spring period, aimed at finding the sentinel animals. The low spatial similarity of positive sentinel chickens to human cases might be due to difficulties in confirming the sentinel status of the specimens sampled.

The integrated surveillance programme was designed to facilitate the early detection (capacity to detect the virus circulation well before the appearance of human WNND cases), but on several occasions, human cases preceded WNV detection in mosquitoes by 7–12 days. In other instances, entomological surveillance made it possible to estimate virus circulation in the range of 10–65 days

Table 4
The vector index (*Culex pipiens*), number and incidence of confirmed neuroinvasive human West Nile cases at the provincial (NUTS2) and district (NUTS3) levels in Vojvodina, Serbia, from 2013 to 2015.

Year	Province/district (NUTS2/NUTS3)	Start VI	Max. VI	Avg. VI	WNVD human cases (n)	Incidence WNND (cases/100,000)
2013	Vojvodina		3.33	1.38	85.00	4.40
	North Banat, Central Banat and South Banat	0.54	3.27	1.91	54.00	8.58
	North Bačka, West Bačka, South Bačka and Srem	2.06	3.33	1.34	31.00	2.38
2014	Vojvodina		1.63	1.15	27.00	1.24
	North Banat, Central Banat and South Banat	0.95	2.56	1.51	14.00	2.23
	North Bačka, West Bačka, South Bačka and Srem	1.24	1.24	0.83	13.00	0.69
2015	Vojvodina		0.62	0.26	10.00	0.52
	North Banat, Central Banat and South Banat	0.30	1.49	0.67	6.00	0.95
	North Bačka, West Bačka, South Bačka and Srem	0.10	1.09	0.39	4.00	0.31

Start VI: seasonal first vector index; Max. VI: seasonal maximum vector index; Avg. VI: seasonal average vector index.

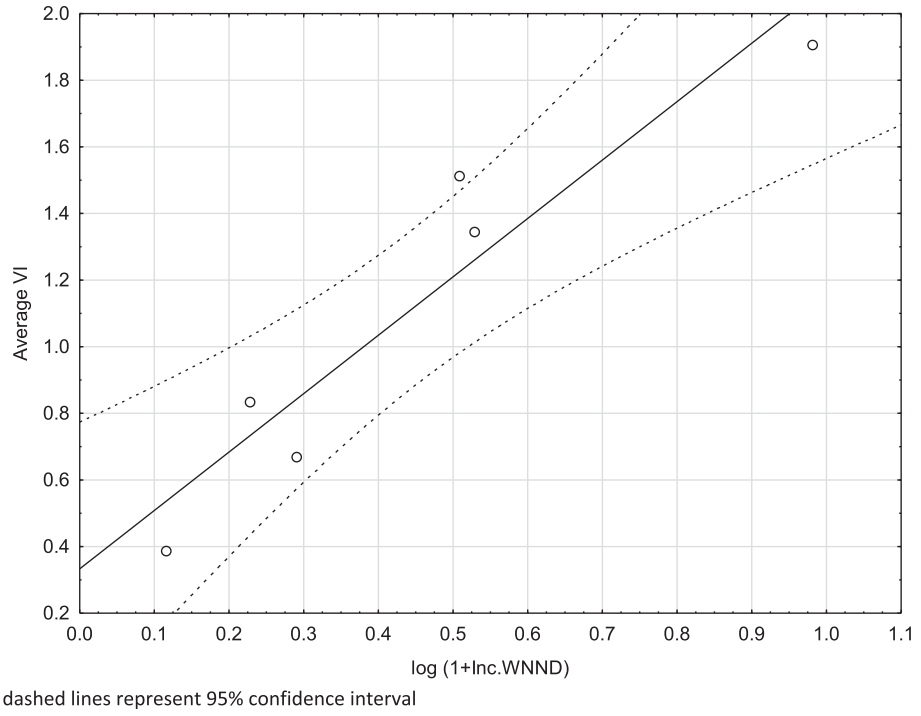

Fig. 3. The correlation between the average vector index values (at the district level in *Culex pipiens*) and the human cases of West Nile neuroinvasive disease in Vojvodina, Serbia, from 2013 to 2015.

before the appearance of the first human WNND cases at the district level. This instability of the system might be the consequence of the small number (50) of *Cx. pipiens* specimens analysed for viral RNA per trap/night sample, which often contained several hundred to several thousand *Cx. pipiens* individuals. The average number of *Cx. pipiens* sampled per night in 2014 was 671, with a maximum of 5617 specimens. The total number sampled in the season was 294,686, and the total number of samples pooled and analysed was 10,884 (3.69%).

The spatial analysis provided evidence of the clustering of mosquito, bird, horse and human cases in specific zones of Vojvodina province and the "unsuitability" for WNV circulation in others. This should be used as an indication for where to focus future epidemiological and ecological studies on WNV transmission.

The integrated surveillance programme in the Emilia-Romagna region of Italy allowed investigators to estimate virus circulation in the range of three to four weeks before the appearance of the first human WNND cases at the province level (corresponding to districts in Vojvodina) and was able to support an evidence-based policy for blood screening, thus avoiding blood unit analyses in the absence of virus, even in areas affected during the previous year [18]. The entomological surveillance in the Emilia-Romagna region was based on the regular testing of up to 200 individuals per trap night (four times more than in Vojvodina), and in the case of large samples, up to 1000 mosquitoes/trap/night/species were submitted to the laboratory for analysis [18]. Theoretically, this provides 4–20 times higher sensitivity and capacity for early detection than the programme in Vojvodina province.

To improve the surveillance system in Vojvodina, the National Veterinary Directorate should consider supporting analyses of more mosquito pools per sample and introducing ornithological surveillance based on corvid species collected in the same seasonal interval as mosquitoes and according to a standard protocol allowing the collection of approximately 1000 specimens per year [18].

Mosquito surveillance should be validated over more seasons, and then might be considered as a valid tool to organize the timely use of personal protection measures and vector control at high risk sites [31]. As in Emilia-Romagna [18], the NUTS3 level seems to be the most appropriate administrative unit for the spatial

organization of the surveillance activities network. This indication is of particular importance for the possible application of a WNV surveillance system for the monitoring of other arboviruses/pathogens/parasites. During the moquito surveillance in Vojvodina province, dry ice baited traps without light (NS2 type) proved to be efficient for the sampling of biting midges, black flies and sand flies. For these reasons, the surveillance of WNV in mosquitoes could be tailored to other situations when/if haematophagous insects other than mosquitoes are transmitting the pathogen.

5. Conclusion

The surveillance system in Vojvodina has shown highly satisfactory results, in terms of the area specificity (the capacity to indicate the spatial distribution of the risk for human cases of West Nile neuroinvasive disease - WNND) and the sensitivity to detect virus circulation even at the enzootic level. The early detection capacity at different administrative levels (NUTS2 and NUTS3) was inversely influenced by the small number of *Cx. pipiens* females analysed per trap per night, combined with a high number of specimens in the sample. The clustering of infected mosquito, horse, bird and human WNND cases in 2014–2015 was highly significant, following the south-west to north-east direction in Vojvodina (NUTS2 administrative level). Human WNND cases grouped best with infected mosquitoes and wild birds. Sentinel chickens showed a limited spatial connection with human WNND. Strong correlations were observed between the average seasonal vector index values and the incidence of human WNND cases recorded at the NUTS3 level. The district level seems to be the most appropriate administrative NUTS (NUTS3) for analyses of the results. At 14 fixed sampling sites (32.56%), WNV was detected in two different (consecutive or alternate) years, while it was found at 2 stations in 3 years and at 1 station in 5 different years, which confirms the endemicity of WNV in Vojvodina. Surveillance in mosquitoes, birds and horses all provided reliable results, in terms of signalling the start of WNV circulation in Vojvodina. More results on the sensitivity of these surveillance systems and a cost evaluation are needed to design the most rational, integrated surveillance to allow the adoption of evidence-based, preventive public health and mosquito control measures.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mcp.2016.10.011.

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