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SEROLOGICAL STUDY ON WNV PRESENCE IN HORSES IN VOJVODINA AFTER THE HUMAN OUTBREAK IN SERBIA IN 2012

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Abstract - To establish the presence of West Nile virus (WNV) infection in the animal population in Serbia after the human WNV outbreak, the presence of anti-WNV IgG antibodies was examined by commercial ELISA of blood sera samples of 130 horses collected in 2012 from 6 stables and 1 settlement in Vojvodina Province, northern Serbia. During the blood sampling, hibernating mosquitoes in the vicinity of the sampled horses were collected (31 pools from 4 locations) and tested for WNV presence by real-time RT-PCR. The presence of anti-WNV antibodies was observed in 49.23% (64/130) horses. Per stable, the percent of seropositive animals ranged from 35% to 64%. All 31 analyzed pools of hibernating mosquitoes tested negative for WNV RNA. The WNV-antibody prevalence of 49.23% obtained in horses during 2012 was much higher than the prevalence (12%) found in horses during 2009/2010. These results, including the confirmed seroconversion in eight horses that tested negative in 2010, indicated an intensive WNV circulation during 2012 in Serbia, and the necessity of implementing surveillance programs.

Key words: WNV, horses, serology, mosquitoes, RT-PCR, Serbia

INTRODUCTION

West Nile virus (WNV) is a mosquito-transmissible *Flavivirus* with zoonotic potential. WNV was first isolated from a febrile woman in the West Nile district of Uganda in 1937 (Smithburn et al., 1940) and today it is considered the most widespread flavivirus in the world, endemic in Africa, Asia, Europe, Middle East, Australia and on the American continent (Komar, 2003; Trevejo and Eidson, 2008; Calistri et al., 2010; Weissenböck et al., 2010; Papa et al., 2011). The disease is indigenous to Africa, the Middle East, Asia and Australia. In 1999 the virus was first reported in New York and spread rapidly throughout the United States and subsequently to Canada, Mexico and Central America; consequently WNV is now endemic in the USA and Canada (Valiakos et al., 2011). In Europe, until the 1990s WNV had caused

sporadic outbreaks with rare reports of encephalitis, but its epidemiological behavior changed when it re-emerged with virulence in Romania, Russia and the Mediterranean basin, causing dozens of human and horse deaths (Castillo-Olivares and Wood, 2004; Blitvich, 2008; Calistri et al., 2010). In addition, only recently the strains of WNV lineage 2 were identified in Europe: in 2004 and 2005 in goshawks and birds of prey in Hungary, in 2007 in Volgograd, Russia, and in 2008 and 2009 in goshawks and a falcon in Austria (Bakonyi et al., 2006; Erdélyi et al., 2007; Platonov et al., 2008; Wodak et al., 2011). Since 2008, WNV has spread widely throughout central and southeastern Europe, constituting a serious veterinary and public health problem for Europe (Ziegler et al., 2012). It is now two years that a strain of lineage 2 has been circulating in Greece (Papa et al., 2011; Valiakos et al., 2011) and Serbia (Petrić et al. 2012; Petrović et al., in press), and in 2011 a WNV RNA belonging to lineage 2 was detected in a human patient in the central part of Italy (Bagnarelli et al., 2011).

WNV infections have been described in a wide variety of vertebrates (Komar et al., 2003). The virus is maintained in an enzootic cycle between ornithophilic mosquitoes, mainly of the *Culex* genus (Hayes et al., 2005; Ziegler et al., 2012), but also *Aedes* and *Ochlerotatus* genera and certain species of wild birds (Savini et al., 2012; Ziegler et al., 2012). WNV was found in more than 150 species of wild and domestic birds (van der Meulen et al., 2005). Wild birds are important to public health because of migration across national and intercontinental borders, making them long-range virus vectors (Linke et al., 2007). Following infection, many bird species produce levels of viremia that are sufficient for transmitting the virus to mosquitoes (Komar et al., 2003). Human and mammals, especially horses, are occasional dead-end hosts and play limited roles in the natural cycle because viremia is generally too low to infect mosquitoes (Dauphin et al., 2004; Valiakos et al., 2011); however, severe neuroinvasive disease and occasionally fatal outcomes can occur.

In horses, WNV infection is frequently clinically unapparent, but around 10% of cases develop neurological disorders with up to 50% mortality rates (Castillo-Olivares and Wood, 2004). The enzootic that involved horses was reported in many countries, such as Canada, France, Hungary, Croatia, Cuba, Morocco, Senegal, Israel, etc. (Rossi et al., 2010). Neurological disorders and deaths in horses in Europe were reported in Italy, Romania and Russia (Blitvich, 2008; Calistri et al., 2010). Lately, an increasing number of severe outbreaks in horses have been reported in Europe, including a large one that took place in northeast Italy in 2008 with 794 cases in 251 equine stables, in which 32 of the serologically positive animals presented clinical signs and five died (Calistri et al., 2010). In 2010, the first outbreak of WNV infection in horses was reported in Spain, with 102 clinical cases and 15 deaths (Garcia-Bocanegra et al., 2011).

The presence and circulation of WNV in Serbia is largely unknown. Only scarce historical data exist about the presence of WNV in human populations and they indicate a seroprevalence of WNV in the republics of former Yugoslavia, as follows: 1-3% in Croatia, 1% in Bosnia and Herzegovina and Kosovo, 1% in Montenegro and 1-8% in Serbia (Vesenjak-Hirjan et al., 1991; Hubalek and Halouzka, 1999). The only serological investigation in Vojvodina was conducted in 1972. Antibodies against WNV were found in 2.6-4.7% of samples in different rural parts of the province (Bordjoski et al., 1972). Recently conducted serological examinations show the presence of anti-WNV IgG antibodies in 18 out of 451 (3.99%) human sera (obtained from 45 patients with viral meningitis or encephalitis and 406 randomly chosen healthy persons) collected from 2005 to 2010 in Vojvodina, with yearly rates varying between 1.97% and 6.04% (Petrić et al., 2012). Also, in the same study, WNV RNA was detected by real-time RT-PCR in 3 out of 841 mosquito pools collected from 2005 to 2010. In August 2012, a clinical outbreak of West Nile virus (WNV) infection in humans was reported for the first time ever in Serbia (EpiSouth Weekly Epi Bulletin - N°232, 2012; ECDC, 2012). Up to 2012 WNV infections in Serbia have never been clinically confirmed. As of November 30, 2012, a total of 70 West Nile fever cases, among which 41 were con-

firmed and 3 fatal (by September 2012), were reported (ECDC, 2012). All the cases were detected in the central and northern parts of the country and among them 72% were reported in the Belgrade district (EpiSouth Weekly Epi Bulletin - N°240; ECDC, 2012). In addition, WNV linage 2 isolates were detected by real-time RT-PCR in 9 wild birds and isolated from one Northern Goshawk during 2012 (Petrović et al., in press).

In our previous study of horses sampled during 2009 and 2010 on the territory of Vojvodina and in areas around the cities of Belgrade and Šabac, we found 12% (42/349) WNV-seropositive animals (Lupulović et al., 2011). This was the first time that WNV circulation was confirmed in the animal population in Serbia. The aim of this study was to assess the WNV seroprevalence in horses in Serbia after the human infection outbreak and to verify the assumption that the presence of WNV infection in horses is one of the indicators of WNV circulation in nature and a forerunner of infection in humans.

MATERIALS AND METHODS

Samples

In order to assess the WNV seroprevalence in horses after the human infection outbreak, blood sera samples from 130 healthy horses, with no clinical sign of disease, of different age and breed, were randomly sampled during November and December 2012. All the samples were collected from horses originating from the territory of Vojvodina from 6 big horse stables (119 samples), and from 11 horses individually reared by different owners in one settlement – Novi Bečej (Table 1 and Fig. 1). Among the examined horses (30 in total), 28 from 4 large stables and 2 individually reared horses from Novi Bečej had previously been examined for anti-WNV antibodies, with 9 positive and 21 negative cases during 2009/2010 (Lupulović et al, 2011) (Table 1). A blood sample was taken from the jugular vein of the horses and in a few hours transported to the laboratory. In the laboratory, blood samples after coagulation were centrifuged and sera were kept frozen at -20°C prior to testing.

The horses were of different sex and age: up to 3 years old (30 animals), from 3 to 5 years old (15 animals), from 5 to 10 years old (54 animals) and older than 10 years (31 animal) (Tables 1 and 3), and of 10 different breeds: 42 English Thoroughbred, 64 Lipizzaner, 10 Nonius, 4 English Half-blood, 15 Serbian Standard-bred, 1 American Standard-bred, 1 Swedish Standard-bred, 1 Holsteiner, 1 Hanoverian, 1 pony and 2 domestic mixed-breed horses (Table 2).

In order to assess WNV presence in virus vectors as the source of infection for the tested horses, during the blood sampling hibernating mosquitoes were collected in stables in the vicinity of the horses. Mosquitoes were collected by entomological aspirator at 4 geographic locations, from 3 large horse stables (stable 1, stable 3 and stable 4), and from a few stables in Novi Bečej with individually reared horses. Mosquito specimens were anesthetized by dry ice, identified to species level on dry ice-cooled paper, pooled according to date, location and species, transported on dry ice to the laboratory and stored at -70°C before testing. Pool size did not exceed 50 specimens. The collected mosquito species and number of analyzed samples are presented in Table 4.

Detection of anti-WNV IgG antibodies

Samples of horse blood sera were tested for the presence of WNV IgG antibodies using a commercial enzyme-linked immunosorbent assay (ELISA) ("Ingezim West Nile Compac", Ingenasa, Spain) following the manufacturer's instructions. All blood sera samples that tested positive by ELISA test were screened for WNV-specific neutralizing antibodies using virus neutralization tests (VNT). VNT was conducted under Biosafety Level 3 conditions on Vero cells using two-fold serial sera dilutions with the WNV NY-99 strain.

WNV genome RNA detection by RT-qPCR

For WNV RNA detection in mosquito pool samples, in house TaqMan-based one-step reverse transcription real-time PCR (RT-qPCR) that amplifies both lineage 1 and 2 strains was used. Viral RNA was ex-

Fig.1 Locations of examined horses on the presence of anti-WNV antibodies in Vojvodina province, Serbia in 2012

Legend to figure 1. Geographical distribution – locations of examined horses in Vojvodina province, Serbia. Locations of examined horse stables are marked with stars and numbers: 1 – horse stable 1; 2 – horse stable 2; 3 – horse stable 3; 4 – settlement Novi Bečej (location of examined individuallz reared horses); 5 – horse stable 4; 6 – horse stable 5 and 7 – location of horse stable 6

tracted using the commercial ISOLATE II RNA Mini Kit (Bioline, The Netherlands) according to the manufacturer's instruction. One-step RT-qPCR was conducted using the commercial kit RNA UltraSense™ One-Step qRT-PCR System (Life Technologies Corporation) with the primers (forward WNproC-F10: 5'-CCTGTGTGAGCTGACAAACTTAGT-3' and reverse WNproC-R153: 5'-GCGTTTTAGCATATTGACAGCC-3') and probe (WNproC-probe 5'-FAM-CCTGGTTTCTTAGACATCGAGATCT -TAMRA-3') that target the nucleocapsid protein C gene regions of both WNV linages 1 and 2, described by Linke et al. (2007). Briefly, 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. 5 µl of nucleic acid extract sample 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

RESULTS

Positive results for the presence of anti-WNV igG antibodies were found in 49.23% (64/130) of the examined horses, and seropositive animals were found in all examined stables. All the ELISA positive results were confirmed by VNT. The obtained WNV seroprevalence in the horses in different stables and/or locations ranged between 35% (7/20) in stable 1 to 64% (16/25) found in stable 3 (Table 1). A high prevalence of WNV-seropositive animals (over 50%) were found in stables located in central and western Bačka areas as well as southern Banat, along the rivers Tisa and Danube (Table 1 and Fig. 1).

Differences in the WNV seroprevalence depended on the age of the horses. The higher WNV seroprevalence was found in animals aged up to 3 years (56.67%) and between 3 and 5 years (60%) than in 5 to 10 year-old (51.85%) and over 10 year-old animals (32.26%) (Table 3). There were no significant differ-

Table 1. Presence of the anti-WNV IgG antibodies in ELISA test in horses per stables and locations

No.	Horse stables / locations	No of examined horses	Gender / Status*	Results of anti-WNV IgG antibody ELISA test			Previously examined horses**	Sero-conversion***
				Positive	% posit.	Negative		
1	Stable 1 Northern Bačka county	20	6 S 4 G 9 M 1 YF	7	35.0%	13	11	1/5
2	Stable 2 Northern Bačka county	21	9 S 5 G 6 M 1 YF	12	57.14%	9	2	0/2
3	Stable 3 South Bačka county	25	10 S 2 G 13 M	16	64.0%	11	12	5/10
4	Settlement Novi Bečej – individually reared horses Central Banat county	11	3 S 8 M	4	36.36%	7	2	0/2
5	Stable 4 South Bačka county	20	14 S 4 YM 2 YF	10	50.0%	10	3	2/2
6	Stable 5 South Banat county	17	7 S 10 M	9	52.94%	8	/	/
7	Stable 6 South Banat county	16	5 S 11 M	6	37,5%	10	/	/
	TOTAL	130	54 S 11 G 57 M 4 YF; 4 YM	64	49,23%	66	30	3/5 stables 8/21 horses that had seroconverted

* S= stallions; G= gelding; M= mare; a; YM= male yearlings and up to 3 years old; YF= female yearlings and up to 3 years old

** horse blood sera samples that have been examined on anti-WNV antibodies with 9 positive and 21 negative results during 2009/2010

*** horse blood sera samples tested positive on anti-WNV antibodies in this study / the same horse blood sera samples that tested negative on anti-WNV antibodies in study conducted in 2009/2010 (Lupulović et al., 2011)

ences between positive animals regarding gender and horse breed.

Of 19 horses from 4 large stables and 2 individually reared horses from Novi Bečej that tested negative for anti-WNV antibodies during the 2009/2010 sampling, 8 horses from 3 large stables showed seroconversion and tested anti-WNV IgG positive in this study (Table 1).

None of the 31 hibernating mosquito pools examined by RT-qPCR for the presence of WNV RNA tested positive in this study, meaning that WNV was not found in the examined virus vectors.

DISCUSSION

In this study we carried out surveillance for WNV infection in a horse population in Vojvodina to exam-

ine the presence of WNV in the environment immediately after the human WNV outbreak in 2012. The blood sera samples were collected during November and December 2012 after the WNV vector activity season. The presence of anti-WNV IgG antibodies was found by ELISA in 49.23% (64/130) horses from 6 of the studied stables and one settlement. The high prevalence of horses with anti-WNV antibodies that in 4 out of 6 stables reached 50% and more, suggests recent and intensive WNV circulation in virus vectors and natural hosts in the area. In our first study conducted on horse sera samples collected during 2009 and 2010, we obtained 12% (42/349) WNV-seropositive animals. Those sera samples were collected from randomly selected horses (often individually reared) originating from the whole territory of Vojvodina Province (28 municipalities) (Lupulović et al., 2011). The results of our present study indicate an increase in WNV seroprevalence from 12% anti-WNV antibody-positive horses sampled during 2009/2010 (Lupulović et al., 2011) to 49.23% positive horses sampled after the WNV vector season in 2012. In support of the theory of very recent WNV infection among the examined horses is the finding that 8 out of 21 horses from 3 out of 5 examined stables or areas, which were negative for anti-WNV antibody presence after the 2009/2010 testing, were found to be WNV seropositive in the present study. The theory of very recent WNV infection is also supported by findings that among young animals up to 3 years old, almost 57% tested positive for anti-WNV antibodies.

In another previous study, we examined the presence of WNV-specific antibodies in 252 horse sera samples collected from 7 different stables and locations in Vojvodina Province and the Belgrade area during 2007-2011 (unpublished). WNV antibodies were found in 72 (28.6%) sera samples. The higher level of 28.6% anti-WNV antibody-positive horses obtained in that study compared to the 12% reported in our first investigation could be explained by the fact that in the first study (Lupulović et al., 2011) the horse sera were collected randomly from the whole territory of Vojvodina, often from horses individually reared. In the second study, the blood sera were taken from horses situated in stables, with a high number of horses in the same location. In a study carried out from 2007-2011 (unpublished data), WNV seroprevalence ranged per stable from 13.3% up to 40% of seropositive animals. The highest prevalence of anti-WNV antibody-positive animals was found in a stable near the Romanian border (40%) and near Belgrade (35.5%). We also examined this stable in the present study (stables 6 and 5 from South Banat County) and observed 37.5% and 52.94% seropositive animals, respectively. The high WNV prevalence found in our previous study (unpublished data) was assumed to be the result of intensive WNV circulation that was confirmed in Romania during 2008-2010 (ECDC, 2011) and the close proximity of the tested horse stables (stable 6), as well as the close proximity of the river Danube with many migratory wild birds (stable 5). In our present study, in samples collected one or two years later from horses in those stables the obtained results show the same situation or slight reduction in WNV seroprevalence among horses in stable 6 (from 40 to 37.5%) near the Romanian border, but an increase among horses in stable 5 (from 35.5% to 52.94%). The location of stable 5 is in the same area where the first human WNV infection clinical cases in the Serbian WNV 2012 human outbreak were detected. About 70% of the total human WNV clinical disease cases in Serbia in 2012 were reported in that area (Belgrade and Pančevo city surroundings), so our results are in strong correlation with the results obtained in the human population, indicating very intensive WNV circulation among virus vectors and probably wild birds as natural hosts of this virus during 2012 and possibly 2011.

The results of horse sera sampled from 2007 to 2011 (unpublished data) from animals in three other stables that we analyzed again in our present study also show a high increase in the prevalence of WNV-seropositive animals from 21.9% to 64% detected in this study in stable 3 and from 14.3% to 50% detected in this study in stable 4. The WNV seroprevalence in horses from stable 1 remained the same (33.3% in the previous and 35% in this study). Both stables (3 and 4) with a high increase in WNV antibody-positive animals are located in South Bačka County

and near the rivers Tisa and Danube. A possible explanation for this high increase could be the close proximity and high density of WNV vector mosquitoes and wild birds and intensive WNV circulation among them. The high prevalence of WNV-seropositive horses that we found in stable 2 (57.14%, and not previously examined) could also be explained in the same manner, as it is also located near the Culex breeding site (small lake). According to the questionnaires used during the sampling, no clinical sign of West Nile diseases was observed in any of the tested horses during 2012. However, the obtained high WNV infection prevalence suggests that health problems exist in the examined stables but that they were overlooked as clinical signs of West Nile disease.

The results obtained in our study are in accordance with recent results obtained on intensive WNV circulation in horses from other European and Mediterranean countries (Calistri et al., 2010; Garcia-Bocanegra et al., 2011). They confirm the high WNV activity in central and southern parts of Europe (Ziegler et al., 2012). Contrary to the results obtained in this study and our previous studies where the prevalence of anti-WNV antibody-positive horses increased from 12% and 28.6%, in Croatia which is west of Serbia, the presence of WNV antibodies was established by IgG and IgM ELISA, and VNT and PRNT in only 72 out of 2098 horse sera (3.43%) collected in 2010 and 2011. The highest seroprevalence was found in eastern Croatia in counties next to the Hungarian, Serbian and Bosni and Herzegovinian state borders (Barbić et al., 2012). In the first study of anti-WNV antibody prevalence in horses in Croatia conducted during 2001 and 2002, only 0.4 (4/980) tested positive (Madić et al., 2003). These data, as well as some other unpublished data on WNV detection and molecular characterization from wild birds and mosquitoes in Serbia, suggest that WNV is spreading from the east and north to the west and south of the Balkan Peninsula. The examined area in our study is a well-known resting and breeding ground for migratory birds on their way from nesting grounds in Europe to wintering areas in Africa. Re-introduction of the virus in the future by birds migrating along this migration route that leads from Europe to Africa should also be considered possible and needs further investigation.

None of the examined mosquito specimen pools tested positive for WNV RNA in this study. The RT-qPCR test used was already confirmed as suitable for WNV detection in mosquitoes through WNV detection in mosquito samples obtained during the summer of 2012 in Serbia (unpublished data). The negative RT-qPCR results on WNV RNA presence in hibernating mosquitoes could be explained by the small number of tested mosquito specimens and the low infection rate within mosquito populations. However, this does not necessarily mean that WNV overwintering in mosquitoes and consequent vertical transmission does not occur.

Finally, we can conclude that our study provides evidence for the recently reported WNV infection among the horses and its intensive circulation of WNV in Serbia. Because of global climate warming, it must be assumed that WNV infections might appear in the future in Serbia. Horses like humans are occasional dead end hosts for WNV. They are also a very good indicator of virus circulation in the area. Positive serological response to WNV infection in a horse population and WNV detection in mosquitoes are the red lights for human outbreaks in the near future. Monitoring disease activity in sentinel animals like horses can provide critical information regarding periods of increased transmission. Surveillance networks involving sentinel animals and mosquitoes have been used in many parts of the world as an early warning system aimed at identifying the periods and locations of elevated risk of WNV disease transmission (Gubler et al., 2003; Angelini et al. 2010). WNV infections are already endemic in other European countries such as Austria, Hungary, Greece and Italy. Therefore, a state-of-the art surveillance system for the detection of incursions of WNV into Serbia is deemed mandatory. Additional epidemiological investigations are currently ongoing.

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