SCIENTIFIC VETERINARY INSTITUTE „NOVI SAD“

INSTITUTE OF VETERINARY MEDICINE OF SERBIA

„One Health – New Challenges“

First International Symposium of Veterinary Medicine

(ISVM2015)

PROCEEDINGS



Hotel "Premier Aqua" - Vrdnik
May 21 – 23, 2015

Publisher

Scientific Veterinary Institute „Novi Sad“, Novi Sad, Serbia

For the Publisher

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Editor in Chief

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Printed by

Multidizajn, Novi Sad

250 copies

Novi Sad, 2015

ISBN: 978-86-82871-36-1

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Invited lecture

WNV IN SERBIA: UPDATE OF CURRENT KNOWLEDGE

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Abstract

West Nile virus (WNV) is a neurovirulent mosquito-borne *Flavivirus* with zoonotic potential. Recently, the number, frequency and severity of outbreaks of infections caused by WNV, with neurological consequences for birds, humans and horses have increased dramatically throughout central and south Europe, including Serbia, constituting a serious veterinary and public health problem.

The emergency of WNV infections in Serbia is described through the current epidemiology situation based on recent data on the presence, prevalence and incidence of WNV infection among virus natural hosts and vectors; sentinel (horses) and other animal species, and in human population. The short overview of the WNV serology studies conducted on horse blood samples collected in different occasions during the last six years, and the results of the serology studies conducted among other animal species like pigs, wild boars, and roe deer in Serbia are presented and discussed. The results of the first studies on WNV presence in mosquito vectors, and in wild birds as virus natural hosts in Serbia are presented and analyzed. Also, the data on the WNV serology studies conducted in human population in Serbia in the last few years, and the existing data of WNV outbreaks in 2012, 2013 and 2014 are included.

In addition, the national program for WNV monitoring launched by the veterinary service in Serbia in April 2014 is presented. The program was funded by the Veterinary Directorate, and it was implemented on the field by veterinary service in collaboration with entomologists and ornithologists. The main objective of the monitoring program was early detection of WNV presence in nature, and consequently timely alerting of human health services and local governments in order to control the mosquito population and to inform the local communities. The monitoring program was based on the direct and indirect monitoring of WNV presence in nature. Indirect monitoring of virus presence was performed by serological testing of WNV seronegative - sentinel horses and backyard chickens hatched during 2014, and direct monitoring was done by molecular testing of WNV presence in pooled mosquito's samples and in wild birds. Number of tested samples is defined at the level of each district of the Republic of Serbia in relation to the risks of WNV infection.

Keywords: West Nile virus, antibody and virus detection, domestic and wild animals, human population, mosquitoes, surveillance program, Serbia

Introduction

West Nile virus (WNV) is a neurovirulent mosquito-borne *Flavivirus* with zoonotic potential, which is maintained in nature in an enzootic transmission cycle between avian hosts and

ornithophilic mosquito vectors. The virus occasionally infects other vertebrates, including humans and horses, in which it may cause sporadic disease outbreaks that may result fatal. West Nile virus (WNV) was first isolated from a febrile woman in the West Nile district of Uganda in 1937 (Smithburn et al., 1940) and today is considered as the most widespread flavivirus in the world, endemic in Africa, Asia, Europe, Middle East, Australia and Americas (Trevejo and Eidson, 2008; Calistri et al., 2010; Weissenböck et al., 2010; Papa 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* genus, and certain wild bird species (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 birds migrating across national and intercontinental borders and becoming a long-range virus vectors (Linke et al., 2007). Following infection, many bird species produce levels of viraemia 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 viraemia is generally too low to infect mosquitoes (Dauphin et al., 2004; Valiakos et al., 2011), however severe neuroinvasive disease and occasionally with fatal outcomes can occur.

In Europe, until the 1990´s WNV had caused sporadic outbreaks with rare reports of encephalitis but its epidemiological behaviour changed when it re-emerged in Romania, Russia and the Mediterranean basin causing dozens of humans and horses deaths (Castillo-Olivares and Wood, 2004; Blitvich, 2008; Calistri et al., 2010). Also, 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 been heavily spreading throughout central and southeastern Europe, constituting a serious veterinary and public health problem for Europe (Barbić et al., 2012; Ziegler et al., 2012).

The history of West Nile fever in Serbia is largely unknown. Due to absence of routine diagnose praxis and limited resources in hospitals of Serbia the human cases of meningoencephalitis of "unknown" origin, that should be submitted to laboratory testing for WNV presence, had been neglected up until 2012. In addition, with the exception for research purpose, the regular, program based WNV surveillance in sentinel chickens, horses or mosquitoes did not exist before 2013. Only scarce historical data exists about the presence of WNV in human population and indicate seroprevalence of WNV in republics of former Yugoslavia of 1-3% in Croatia, 1% in Bosnia and Herzegovina and Kosovo, 1% in Montenegro and 1-8% in Serbia (Vesenjak-Hirjan et al., 1991).

WNV epidemiology in human population in Serbia

First serological investigation was conducted in 1972 and antibodies against West Nile virus (WNV) were found in 2.6% - 4.7% of human sera (Bordjoški et al., 1072). After almost a 30 years gap, ELISA IgG testing revealed the seroprevalence of WNV in 6.67% of human sera of the 45 patients who were hospitalized for encephalitis or meningoencephalitis in the period 2001-2005 and 3.69% of 406 samples taken from healthy persons. Average seroprevalence of WNV in samples taken from 2001-2010 was 3.99% (18 out of 451). A total of 337 persons tested in 2010 were exposed to at least one mosquito exposure related risk factor. Among them, 5.04% were seropositive to WNV. Most of the probably infected people did not screen windows and doors in their houses, while in the group using window screens only 0.88% were seropositive to WNV (Petrić et al., 2012). The obtained seroprevalence in this study still didn't suggest more intensive circulation of WNV in Serbia. Except this data, as to our knowledge, no clinical manifestation of disease was ever reported in Serbia until 2012.

In August 2012, an outbreak of WNV infection in humans, was reported for the first time ever in Serbia (EpiSouth Weekly Epi Bulletin - N°232, - N°240; ECDC, 2012; Obrenović et al., 2013; Popović et al., 2013), being the first time that WNV infections in the country have been associated with clinical symptoms. As of November 30, 2012, a total of 71 West Nile fever cases were reported, among which 42 were clinically and laboratory confirmed, and in 9 cases resulted fatal (lethality of 12.7%). All the cases were detected in central and northern part of the country, 72% of them in the Beograd district (ECDC, 2012; Obrenovic et al., 2013; Popovic et al., 2013). This epidemic continued, and became even more severe during 2013. As of November 2nd, 2013, a total of 303 West Nile fever cases were reported, among which 202 were clinically and laboratory confirmed, and 103 were classified as probable cases. Infection in 35 cases resulted fatal (lethality of 11.6%). Almost all of the cases were also detected in central and northern part of the country (Institute of Public Health of Serbia, 2014).

The epidemic also continued during 2014. The outbreak characteristic was similar as those from 2012. In total 76 clinical cases were reported and 9 cases resulted fatal. Almost all the cases were detected in central and northern part of the country, and 65 out of 76 (86%) of them in four counties (Belgrade, South Banat, South Backa and Srem county) (ECDC, 2014). In Europe during 2014 in total 163 human cases were reported, so 47% of cases were from Serbia. It can be concluded that WNV infection in Serbia become endemic and can be assumed that it will be public health problem in the coming years also.

WNV epidemiology situation in horses

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 tested horses from northern part of country, sampled during 2009-2010, presented specific neutralizing WNV antibodies (average PRNT90 = 120, range: 42–650), which in one case also cross-neutralized Usutu virus. This was the first time that anti-USUV high neutralizing antibody titers have been reported in horses. Positive horses were found in 14 of the 28 municipalities studied, which are up to 200km distant (Lupulović et al., 2011). In our another previous study (Medić et al., 2014), the presence of WNV specific antibodies was examined in 252 horse sera samples collected from 7 different stables and locations in Vojvodina province and Belgrade area, during 2007-2011. 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 comparing to 12% reported in our first investigation could be explained by the fact that in the first study the horse sera were collected randomly from the whole territory of Vojvodina province, often individually reared, and in the second study the blood sera were taken from horses situated in the stables, with high number of horses in the same location. Also, most of the examined horse sera in this study were sampled during 2010 and 2011 that could imply on possible more intensive WNV circulation during year 2011. WNV seroprevalence ranged per stable from 13.3% up to 40% seropositive animals. The highest prevalence of anti-WNV antibody positive animals was found in stable near Romanian border (40%) and near Belgrade (35.5%). The high WNV prevalence was assumed to be the results of intensive WNV circulation that was confirmed in Romania during 2008 - 2010 and the close proximity of river Danube with a high circulation of migratory wild birds (Medić et al., 2014).

In addition, to asses WNV presence in the environment immediately after the human WNV outbreak in 2012, during November and December of 2012, presence of anti-WNV IgG antibodies were examined by commercial ELISA test in blood sera samples of 130 horses from 6 stables and 1 settlement in Vojvodina province, northern Serbia (Petrović et al., 2014). Positive results were obtained in 49.23% (64/130) samples. Per stable, percent of seropositive animals was from 35% to 64%. This prevalence (49.23%) obtained in horses during 2012 was much higher than that found in horses during 2009 and 2010 (12%), including the confirmed seroconversion in at least 8 horses that

tested negative in 2010, thus confirming an intensive WNV circulation in 2012 on the territory of Serbia. Very recent WNV infection theory is also supported by findings that among young, up to 3 years old animals, almost 57% tested positive on anti-WNV antibodies (Petrović et al., 2014).

Similarly, 96 horses from 5 tested stables during 2012 were tested again during 2013 with the same methodology. High prevalence of 46.88% (ranged between stables from 23.53-75.0%) with new cases of seroconversion were detected also indicating an intensive WNV circulation in 2013 (unpublished data). The very recent data suggests that more than 50% of horses from Northern and central part of Serbia, the areas around the rivers Danube, Sava and Tisza, are seropositive (unpublished data).

Presence of WNV in natural host species and vectors in Serbia

Presence of WNV was also studied in susceptible wild bird's species and mosquitoes as virus natural hosts and vectors in the last few years in Serbia. WNV circulation was examined by ELISA and PRNT in 92 blood sera and 81 pooled tissues from 133 wild resident and migratory birds from 45 species within 27 families collected from January until September 2012 in Vojvodina Province - northern part of Serbia (Petrović et al., 2013). WNV antibodies were detected in 7 (7.6%) blood sera of: 4 Mute swans (*Cygnus olor*), 2 White-tailed eagles (*Haliaeetus albicilla*), and 1 Common pheasant (*Phasianus colchicus*). Three of the seropositive birds found here were resident birds (two White-tailed Eagles and one Common Pheasant), while the other four (Mute Swans) are considered both migratory and resident birds in Serbia. Viral RNA was detected, for the first time in Serbia, by RT-qPCR in 9 birds: 3 Northern goshawks (*Accipiter gentilis*), 2 White-tailed eagles, 1 Legged gull (*Larus michahellis*), 1 Hooded crow (*Corvus cornix*), 1 Bearded parrot-bill (*Panurus biarmicus*), and 1 Common pheasant. Seven of these birds died during the summer of 2012 while two (a pheasant and one goshawk) died during winter-early spring. Eight of the nine WNV RNA positive birds were strictly resident, suggesting that they became infected in the country. Moreover, isolation of WNV-RNA from dead predators (5 of the 9 WNV positive birds) provides more evidence that birds of prey play a key role in virus transmission (Petrović et al., 2013).

All the isolates were classified by phylogenetic analysis of partial E region sequences as lineage 2 WNV strains and they were closely related to those responsible of recent outbreaks in Greece, Italy, and Hungary. Further on, West Nile virus from one Northern goshawk (SRB-Novi Sad/12) was isolated on Vero cell culture and its full genome sequenced. Phylogenetic analysis of this complete genomic sequence showed a lineage 2 strain that clusters with the viruses responsible for the most recent human and animal outbreaks reported in neighbouring countries, however, SRB-Novi Sad/12 isolate was unique, as it showed a total of 29 distinctive nucleotides when compared to those circulating in Europe. Comparison of partial sequences of the E region from five additional WNV sequences recovered from respective birds in this study shows that at least two different groups of lineage 2 strains, which simultaneously circulated during summer of 2012, can be distinguished (Petrović et al., 2013). These results suggest that WNV has reached the country in, at least, two different events and suggest that the virus not only has become endemic in Serbia and surrounding countries, but that it is also evolving while circulating in the area. According to these findings, it seems logically to think that since its original detection in Hungary, WNV lineage 2 has expanded southwards and reached Serbia recently, but it cannot be ruled out that there had been prior sporadic human and animal cases that have gone unnoticed.

Mosquitoes were sampled on the spots where possible circulation, based on serological testing of humans and horses, was detected in order to optimally utilize available number of test kits and minimize the number of pools for mosquito surveillance. Data obtained from human serological surveillance in 2009 had indicated seven "hot spots" of possible WNV transmission in the municipality of Novi Sad (capital of Vojvodina Province). In 2010 mosquito trapping was focused

on these "hot spots" revealing WNV RNA in three out of 50 pools of *Cx. pipiens pipiens*, the first finding of WNV in mosquitoes in Serbia (Petrić et al., 2012). During this experiment, a total of 56757 mosquitoes (841 pools of 50 individual insects) originating from 66 localities in 29 settlements in Vojvodina were examined. The presence of WNV genome was established in only three pooled-samples of mosquitoes collected during 2010 in the territory of Detelinara (part of the city of Novi Sad). The isolate was typed as lineage 2 WNV (Petrić et al., 2012). During the year 2012 WNV outbreak in Serbia, mosquitoes were collected at 62 sites in 31 municipalities in Serbia. West Nile virus RNA was detected in 9.55% of 314 mosquito pools (11113 specimens) from 9 municipalities in *Cx. pipiens pipiens*, *Aedimorphus vexans* and *Culiseta annulata* (Petrić et al., *in press*). In addition, presence of WNV genome was confirmed in 28 (9.2%) out of 306 mosquito pools collected and tested from 20 localities in Vojvodina Province of Serbia during 2013 (unpublished data). The very recent testing of WNV presence in mosquito vectors (*Cx. pipiens pipiens*) was done during the first WNV surveillance program during 2014 in Serbia. The *Cx. pipiens pipiens* mosquitoes were collected from the whole territory of Serbia and out of 995 tested mousquito pools WNV were found present in 22 (2.21%) samples (unpublished data). These data point on slightly less intensive WNV circulation during 2014 in Serbia, but still the virus circulation in vectors is endemically present for the least four last years.

WNV seroprevalence in different domestic and wild animal species

Presence of anti-WNV antibodies in blood sera of different animal species detected recently also represents the evidence of intensive circulation of WNV in the last few years in Serbia. Out of tested blood sera of 66 donkeys, 1076 dogs, 318 poultry, 102 sheep, 6 goats, 30 cattle, and 5 deer, collected between 2008 and 2012, presence of anti-WNV antibodies was found in 0.93% dogs and 0.31% poultry (Đuričić et al., 2013). To assess WNV circulation among mammals in the country, 688 samples obtained from 279 farm pigs, 318 wild boars, and 91 roe deer were investigated for the presence of antibodies to WNV by ELISA and viral neutralization test. ELISA-reactive sera were identified in 43 (15.4%) pigs, 56 (17.6%) wild boars, and 17 (18.7%) roe deer. Of these, 6 (14%), 33 (59%), and 4 (23.5%) respectively, neutralized WNV. One out of the 45 ELISA negative sera tested, from a roe deer, neutralized WNV (Escribano-Romero et al., 2015).

Methodology of WNV surveillance program in Serbia

Veterinary Directorate of the Ministry of Agriculture and Environmental Protection infront the Veterinary Service launched and funded the national WNV surveillance program starting from April 2014. The surveillance program encompassed sentinel species (poultry and horses), mosquitoes (particularly species *Culex pipiens*, which were confirmed as most prevalent WNV vectors in our region) and wild bird species, which are natural virus reservoirs and populate the natural habitats in Serbia, either temporarily or permanently.

The surveillance program was conducted throughout the year according to the provided guidelines. Active surveillance was performed by serological examination (by ELISA) of sentinel poultry and horses and by testing of virus presence in samples of mosquito vectors (sampled by dry-ice baited traps in the period of most prominent vector activity using special traps), as well as in the samples of all collected dead wild birds belonging to the species susceptible to WNV (tested throughout the year). The detection of virus presence in birds and mosquitoes was done by molecular diagnostic (RT-PCR or real-time RT-PCR). Passive surveillance encompassed serological (testing of paired serum samples) and virological examination of clinically ill horses manifesting signs of CNS dysfunction.

The active and passive surveillance encompassed all municipalities in the Republic of Serbia. The selection and distribution of sampling localities in each county-region is defined by epizootiological services of Scientific and Specialized Veterinary Institutes according to the assessment of the risk of exposure to WNV (according to the human cases in the last 2 years, and previous studies performed in horses). The whole Program was described in details by Petrović et al. (2014a). The basic methodology of WNV surveillance program in Serbia in 2014 is described in the Table 1.

Table 1. WNV surveillance program (sampling /testing) in Serbia in 2014 (Petrović et al., 2014a)

	High-risk regions/Counties	**Lower-risk regions/Counties**
1. Testing of sentinel animals (domestic poultry and horses) aimed at early detection of WNV circulation		
Surveillance of sentinel poultry on rural households – poultry hatched in current year (backyard poultry)	Serological testing at the authorized institute in the period May-September from 10 settlements / County; 5 samples / settlement from et least one household according to described schedule. 6 samplings (1 in May; 1 in June; 2 in July; 1 in August – by middle; 1 in September (until 15 Sept)	Serological testing at the authorized institute in the period June-September from 6 settlements / County; 5 samples / settlement from et least one household according to described schedule. 4 samplings (1 in June; 1 in July; 1 in August – by middle; 1 in September (until 15 Sept)
Surveillance of sentinel horses	Serological testing of 50 sentinel horses in the authorized institute, sampling from minimum 3 localities per County. Sampling and blood testing of same horses to be performed three times (in three occasions) (June-July-August)	Serological testing of 30 sentinel horses in the authorized institute, sampling from min 3 localities per County. Sampling and blood testing of same horses to be performed three times (in three occasions) (June-July-August)
2. Testing aimed at early detection of WNV in natural reservoirs and vectors		
Virus surveillance in wild birds	Application of *RT-PCR* or *real time RT-PCR* methodology for testing samples of dead susceptible bird species throughout the year, or up to 100 samples of purposely hunted birds or live captured susceptible bird species per County during the period May - October	*RT-PCR* or *real time RT-PCR* methodology for samples of up to 50 dead birds (WNV-susceptible species) per County during the period May - October
Virus surveillance in vectors - mosquitoes (*Culex pipiens*)	Collecting mosquitoes at 2-week intervals in the period May-September at 10 localities within the County and testing the virus presence by *RT-PCR* or *real time RT-PCR* methodology (7 samplings in the period from end May to the first half of September)	Collecting mosquitoes at monthly intervals in the period May-September at 5 localities per County and testing the virus presence by *RT-PCR* or *real time RT-PCR* methodology (5 samplings once a month in the period from second half May to the first half of September)

Future steps and expectations

The aforementioned serological and virological examinations confirmed active circulation and endemic presence of WNV in the territory of the Republic of Serbia. Based on the obtained results and anticipated intense circulation of WNV that poses substantial risks for both public and animal

health in Serbia, there is a need for further studies and continuous monitoring and surveillance of WNV infection and virus epidemiology in Serbia in the coming years.

Acknowledgments

This work is conducted within the project TR31084 funded by the Serbian Ministry of Education, Science and Technological development

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