

West Nile virus surveillance in humans and mosquitoes and detection of cell fusing agent virus in Vojvodina province (Serbia)

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Abstract

After the large 1996 outbreak of West Nile virus (WNV) in Romania and spreading from East (1999) to the West Coast (2002) of USA at a remarkable speed, WNV has, in 2010, reemerged in Europe causing outbreaks in human populations reported from Greece, Hungary, Italy, Romania and Russia. Surveillance of WNV in humans and mosquitoes by enzyme-linked immunosorbent assay, rapid antigen panel assay, virus isolation in Vero cells and real time polymerase chain reaction assay was carried out from 2005 to 2010 in Vojvodina Province, northern Serbia. Human sera samples were collected from 27 persons with viral meningitis or encephalitis disease history or from 18 persons with acute viral meningitis or encephalitis and 406 randomly chosen healthy persons from Vojvodina Province. Mosquitoes were sampled in bird reservoirs and human settlements by dry ice-baited traps. IgG antibodies were found in 18 out of 451 (3.99%) human sera with yearly rates varying between 1.97% and 6.04%. Viral RNA was detected in 6 out of 841 pools made out of 56757 sampled, identified and pooled mosquito specimens. In three pools of *Aedes vexans* sampled in 2005, RNA sequences related to cell fusing agent virus (CFAV) were detected. Data obtained from serological surveillance in 2009 were used to indicate "hot spots" of possible WNV transmission in urban area of Novi Sad (capital of Vojvodina Province) where mosquito trapping was focused during the next year. In 2010, three out of 50 pools of *Culex pipiens* sampled in "hot spot" areas were

positive for WNV RNA. Both CFAV and WNV detections in mosquitoes are the first records of such type in Serbia.

Key words: West Nile Virus (WNV), Cell Fusing Agent Virus (CFAV), mosquitoes, seroprevalence

Introduction

The West Nile virus (WNV), an RNA arbovirus (*Flaviviridae*, *Flavivirus*) is the most extensively distributed flavivirus of the Japanese Encephalitis Serocomplex group worldwide. The virus was first isolated in 1937 in West Nile district of Uganda in the blood of a woman with neurologic disorders (1). Over a hundred wild and domestic birds species may serve as reservoir of WNV from which is transmitted by mosquito vector species. Mosquitoes of the genus *Culex* are generally considered the principal vectors of WNV worldwide (2). A broad spectra of mammalian species including man, horse, cat and rabbit can be infected naturally or experimentally with WNV (3). Humans are dead end hosts, unsuitable for further transmission by mosquitoes because of low virus titer and short duration of viremia. Most human infections are asymptomatic. Clinical manifestations can range from uncomplicated febrile illness to fatal meningitis or encephalitis (4). Severe neurologic cases have been reported in around 1% of infected (5).

The presence of WNV is registered in the western Mediterranean and southern Russia in early sixties of the last century (6). The significance of

WNV is not only in the fact that there has been expansion into new areas but also in the changes of the virulence of the virus that has been registered in recent outbreaks. Mild cases of fever, dominant in previously described epidemics have been replaced by outbreaks of cases with severe neurological manifestations and deaths. In USA, the virus was first registered in 1999 when it led to an epidemic of fatal encephalitis in 12% of patients (7). Since then as of end of 2010, 30600 clinically manifested human infections, 12668 cases of meningitis/encephalitis and 1206 fatalities have been reported to CDC (8).

Among the outbreaks registered in recent decades in Europe, encephalitis epidemic in southeastern Romania in 1996 was the first large urban outbreak (9). The activity of the virus remained in Romania after the epidemic. From 1997 - 2000 there were 39 cases of human infection and 5 (13%) deaths among them (10). In 2010 a total of 57 cases of WNV infection were identified in Romania with the case fatality rate of 8.8% (11). The same year the outbreak of WNV infections in humans in Greece (12) is another timely reminder that WN fever is a reemerging vector borne disease in Europe.

The incidence of West Nile fever in Serbia is largely unknown. Only scarce historical data obtained by hemagglutination inhibition test 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 (6). Only serological investigation in Vojvodina Province was conducted in 1972 and antibodies against West Nile virus were found in 2.6% - 4.7% of samples in different rural parts of province (13). To our knowledge, no West Nile virus RNA was detected in mosquitoes on the territory of both former Yugoslavia and Serbia.

The aim of this study was both to reestablish surveillance for WNV specific antibodies in the human population in Serbia applying enzyme-linked immunosorbent assay (ELISA) and to explore the presence of viral RNA in possible mosquito vectors by virus isolation in Vero cells, rapid antigen panel assay and real time polymerase chain reaction (RT-PCR) test.

Materials and methods

Vojvodina is a province in northern Serbia bordering with Croatia on the west, Hungary on the north and Romania on the east. It is a southern part of Panonian plane, rich in rivers (the Sava, the Danube and the Tisa) and covered typically by agricultural land. The climate is temperate, average yearly temperature 11°C, average monthly minimum January 1.3°C, maximum July 21.4°C, mean yearly rainfall 658 mm.

ELISA testing, calculation and interpretation of results, for IgG determination, were performed according the instructions of manufacturer (Euroimmun AG) of the Euroimmun Analyzer I-2P. Results were evaluated semi quantitatively by calculating a ratio of the extinction value of serum sample to the extinction value of the calibrator 2 which was included into the test. Results were considered as positive (ratio equal to or greater than 1,1), borderline (ratio between 0,8 and 1,1) or negative (ratio less than 0,8). All borderline samples (5) were grouped negative for interpretation of the results.

First attempt to investigate the presence of WNV antibodies in humans was performed in a sample of 27 patients (aged 11 to 68, from 4 municipalities in Vojvodina – Ada, Beocin, Novi Sad, Novo Milosevo) who were hospitalized at Clinic for Infectious Diseases Novi Sad for encephalitis or meningoencephalitis in the period 2001-2005. During September and October 2007, serum samples were collected from 152 randomly chosen healthy persons from 4 municipalities of Vojvodina (Backa Palanka, Beocin, Kisac, Novi Sad). Sera consisted of 101 males and 51 females, aged 18-74 years, were tested for IgG antibodies. In 2009, 182 inhabitants from Novi Sad with no history of neurological diseases (72 children aged from 1 to 18, 34 boys and 38 girls, and 110 adults aged from 19 – 84, 105 females and 77 males) were tested. In the study conducted in 2010, 72 sera samples of randomly chosen healthy person and 18 patients with meningoencephalitis from Novi Sad were tested.

All persons included in the study were interviewed about possible exposure to mosquito bites. Type of housing, proximity to mosquito breeding sites, length of time spent outdoors, presence of window/door mosquito screens, use of mosquito repellents, presence of mosquitoes in the home

and fever/neurological disease history were considered as the risk factors and possible indicators (the last one) of WNV infection.

Mosquitoes were collected from June to October by dry ice-baited NS-2 trap (14) at 66 sampling locations distributed in 29 settlements of Vojvodina province (Ada, Backo Gradiste, Backo Petrovo Selo, Banatsko Novo Selo, Becej, Bela Crkva, Begec, Beocin, Bukovac, Coka, Futog, Glogonj, Hajdukovo, Jabuka, Kanjiža, Kovilj, Kupinovo, Mol, Novi Kneževac, Novi Ledinci, Novi Sad, Novo Milosevo, Palic, Pancevo, Petrovaradin, Rakovac, Sremska Kamenica, Starcevo and Subotica) within the area ranging from 44°42'12" to 46°06'06" North and 19°37'14" to 21°25'01" East. From 2005 to 2009 sampling sites were chosen according to hypothetically higher probability for virus occurrence eg. migrating birds resting places, high abundance of *Culex* spp. mosquitoes. In 2010, traps were placed in "hot places" identified by the previous year serological surveillance in human population. Specimens sampled were anesthetized by dry ice, identified to species (15,2) 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 for rapid antigen panel assay (VecTest®) did not exceeded 50 mosquito specimens while for virus isolation in Vero cells, and RT-PCR maximum number was 200 specimens per pool.

Rapid antigen panel assay (VecTest®) and virus isolation in Vero cells were performed according to manufacturer instruction and standard procedure (16). After being processed for virus isolation, mosquito cultures were inoculated into Vero cells and examined daily for evidence of viral cytopathic effect (CPE) for 7 days.

From 2005-2009, 61 mosquito pools were tested by rapid antigen panel assay (VecTest®). Additional 730 were screened for cytopathic effect on Vero cell. Positive and suspect samples (115 pools) were further analyzed by generic RT-nested-PCR for detection of flaviviruses (17). In 2010 when WNV was circulating in surrounding areas the scheme was changed, and 50 pools were analyzed by specific RT-PCR in mosquitoes sampled at hot spots only.

For detection of viral RNA in 2010, pools were homogenized in sterile phosphate buffer and centri-

fuged at 13 000 rpm for 1 minute. The WNV Real – TM (Sacace Biotechnologies S.r.l., Como, Italy) kit was applied according to the instructions provided by the manufacturer. In order to control the process of isolation Internal Control, Negative Control and Pos WNV-RNA-rec control were applied. For control of amplification RNA-buffer and cDNA-WNVc were used. RT PCR was performed using Applied Biosystems 7500 instrument.

Results

ELISA IgG testing of human sera revealed the seroprevalence of WNV in Vojvodina (Serbia) and determined the frequency of persons who were probably in contact with the WNV regardless of the time when the contact occurred. Considering the results of ELISA IgG tests (Table 1), 15 out of 406 healthy persons (3.69%) and 3 out of 45 persons who suffered from viral meningoencephalitis (6.67%) were seropositive for WNV antibodies. Average seroprevalence of WNV in samples taken from 2001-2010 was 3.99% (18 out of 451).

Table 1. Seroprevalence of WNV in persons from Vojvodina (2001-2010)

Status	IgG positive (ratio ≥1,1)	IgG negative* (ratio <0,8)
Healthy (N=406)	15 (3,69%)	391(96,31%)
Meningoencephalitis in the past or acute meningoencephalitis (N=45)	3 (6,67%)	42 (99,33%)

* all borderline samples (5) with ratio $0,8 \geq n < 1,1$ were treated negative

IgG ELISA of sera of the 27 patients who were hospitalized for encephalitis or meningoencephalitis in the period 2001-2005 revealed presence of WNV antibodies in two samples (7.41%), a woman aged 36 from Ada and an 11 years old boy from Novo Milosevo. In 2010, IgG antibodies were found in 1 of 18 patients with viral meningoencephalitis (5.55%). During 2007, IgG antibodies to WNV were found in 3/152 serum samples (1,97%) obtained from Backa Palanka, Kisac and Novi Sad and in 2009 in 11 out of 182 healthy persons (6.04%) with no history of neurological diseases. Throughout 2010 IgG antibodies to WNV were found in 2/90 (2,22%) serum samples (1/78

random and 1/18 with viral meningoencephalitis history) samples taken from inhabitants of Novi Sad. Sex and age distribution of seropositive persons was slightly shifted to males and age group above 35 years (Table 2).

A total of 337 persons were exposed to at least one risk factor (Table 3). Among them, 5.04% were seropositive to West Nile virus. Most of the probably infected people did not screen windows and doors in their houses, either alone or combined with one of the other risk factors. In the group of persons who denied exposure to any of risk factors only 0,88% were seropositive to West Nile virus.

Table 2. Distribution of seropositive (ratio ≥ 1.1) persons according to sex and age (2001–2010)

Age group (years)	Males	Females
< 18	4	1
18-25	2	1
26-35	0	0
≥ 36	5	5
Total	11	7

Table 3. Seroprevalence of WNV in persons from Vojvodina by risk factors (2001-2010)

IgG against WNV	Risk factor/s	
	Present Number (%)	Absent Number (%)
Seropositive	17 (5,04)	1 (0,88)
Seronegative*	320 (94,96)	113 (99,12)
Total	337 (100)	114 (100)

* all borderline samples (5) with ratio $0,8 \leq n < 1,1$ were treated negative

During six years of surveillance, a total of 56757 mosquitoes belonging to 15 species (*Anopheles claviger*, *An. hyrcanus*, *An. maculipennis*, *An. plumbeus*, *Aedes cinereus*, *Ae. rossicus*, *Ae. vexans*, *Ochlerotatus annulipes*, *Oc. caspius*, *Oc. dorsalis*, *Oc. sticticus*, *Coquilletidia richiardii*, *Culex modestus*, *Cx. pipiens*, *Culiseta annulata*) were sampled by dry ice baited traps and separated into 841 pools.

None of 176 pools of mosquitoes sampled in 2005 and processed by VecTest® (61 pools) or on Vero cells (115 pools) was WNV positive but positive results after RT-PCR for flavivirus amplification were obtained in 3 pools, sequences being related to cell fusing agent virus (CFAV).

All three pools were sampled at Vojvodina northernmost location, Ludasko lake near Hajdukovo (46°06'06" N and 19°49'53" E) and made of *Ae. vexans* mosquitoes.

No WNV was found in the 615 pools processed on Vero cells between 2006 and 2009. Data obtained from 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 to previously identified "hot spot" streets, city districts and suburbs. Three out of 50 pools of *Cx. pipiens* sampled in one of seven identified "hot spot" areas (within the perimeter 1 km, Detelinara borough of Novi Sad - 45°15'18" N, 19°50'41"E) were positive for WNV RNA.

Both CFAV and WNV detections in mosquitoes are the first records of such type in Serbia.

Discussion

Surveillance of WNV in Serbia is characterized by more than a 30 years long gap, last survey being published by Bordjoski et al. (13). Despite the epidemic in neighboring Romania in 1996 (9) surveillance of WNV has continued to be neglected until this work. Only recently surveillance in birds, mosquitoes and horses had been conducted (18,19).

Serological findings throughout 2005 to 2009 showed that WNV is probably present in Vojvodina. First positive sera were obtained from patients who were hospitalized for viral encephalitis or meningoencephalitis of uncertain etiology during a five-year (2001-2005) period, IgG seroprevalence to WNV being 7.41%. In serum samples taken from 105 patients from Serbia, occupationally exposed to mosquito bites, IgG antibodies to WNV were confirmed in 4,76% by indirect immunofluorescence assay (20) while during this work IgG antibodies were detected in 5.04% of blood samples from people exposed to one or more WNV risk factors (most of them without window/door screens). Average prevalence of IgG antibodies in human sera registered during our survey was 3.99%, with yearly rates varying between 1.97% and 6.04%. In different parts of Italy the incidence of hemagglutinin inhibition antibodies to WNV ranged from 2 to 23%, in Greece from 1 to 27%, in Bulgaria 3%, in Slovakia from 1 to 4% and in

Austria from 1 to 6% (6). In Hungary, 5312 persons were examined and 30 (0,56%) had antibodies against WNV (21). One study carried out in Germany included 14437 blood donors tested by ELISA IgG test for WNV and revealed that 5.9% were initially anti-WNV reactive (22).

The incidence of antibodies in the tropics was higher than that reported for Europe. In Ghana seroprevalence of IgG antibodies to WNV ranges from 4,8% in children to 27,9% in adults. The same study found that 2,4% of children had IgM antibodies against this virus (23). In a similar study, IgG antibodies to WNV ranged from 18,4% in children under 14 years of age to 51,7% in patients aged over 44 (24). In this work, seroprevalence in children was 6.94% (5/72), and 9.09% (10/110) in samples from people over 36 years of age. No considerable bias of the cases concerning gender of individuals tested within older age groups (above 18) was observed, but probable infections were diagnosed in more boys (80%) than girls (20%), the ratio not being biased by the sample size.

During the last decade, human infections with WNV were registered in France, Germany, Denmark and Scandinavia (25,26,27,28), with an increasing trend of number of patients with WNV infections of central nervous system in northeastern Italy and Hungary (21,29). In 2010 major outbreaks of WNV infection in Europe were registered in Greece, Romania and Russia with the case fatality rate of 13.3%, 8.8% and 2.60% respectively (11,12,28,30). Small genetic changes in viral RNA can significantly enhance virulence to humans, possible scenario of the outbreak of WNV lineage 2 in 2010 in Greece. Lineage 2 WNV strains were previously thought to be of low virulence. Nevertheless recent studies in South Africa suggest that lineage 2 WNV strains are a cause of neurological disease in horses and humans (31). "Parental" WNV lineage 2 strain was of much lesser virulence in Hungary and Austria (32,33) and then after registered change by one of (neuro-) virulence and pathogenicity markers (Nowotny personal communication) suddenly induced outbreaks in human population in Greece in 2010. Sequencing of viral RNA found in *Cx. pipiens* mosquitoes in Novi Sad is in progress, and up to now it is proven that it belongs to WNV lineage 2 strain.

Serologic research indicated the likely presence of West Nile virus in Vojvodina. However, former attempts to detect the virus in mosquitoes in Vojvodina and the other parts of Serbia were not successful (18). In this work, RNA of WNV was detected in urban *Culex pipiens* mosquitoes. Many other species of the genus *Culex* are involved in WNV transmission worldwide (34,35,36). The proliferation of the probably main vector, *Culex pipiens*, in certain parts of Europe (12), the increasing virulence of the virus and the recent outbreaks of WNV infections in humans in USA and Greece are timely reminders that WN fever is an important reemerging vector borne disease in Europe.

Detection of a flavivirus sequence similar to CFAV in *Ae. vexans* was the second record of similar viruses in Europe, after Spain from 2001 to 2004. The flavivirus sequences identified in Spain (37,38) were different from all known Flavivirus mosquito viruses, but very close to Kamiti River virus or cell fusing agent virus.

Currently, the routine diagnosis of WNV infection in humans is not carried out in Vojvodina. The incidence of clinically manifested human cases is not known. According to the results of this study the virus is present in Vojvodina and circulates among urban mosquitoes and likely humans. The introduction of diagnostics of WNV infection in regular medical practice is needed in order to establish the system for accurate diagnosis and consequently more efficient treatment of serious clinical manifestation of WNV infection. Due to absence of routine diagnose praxis and limited financial resources in hospitals of Serbia the human cases that should be submitted to RT-PCR tests during the infection are overlooked. In addition, the regular sentinel chickens and mosquito surveillance does not exist. Therefore, system of searching for the virus and its detection in Serbia have been set backwards, based on serological testing of humans in order to optimally utilize available number of test kits (restricted by funding) and minimize the area (and costs of kits) for mosquito surveillance. When grouping of the IgG positive people (likely cases) is detected, "hotspots" for sustainable virus surveillance in mosquitoes were defined in order to detect virus, raise the awareness of public health officials and prevent probable outbreaks and future infections in humans and horses.

Conclusion

Currently, the routine diagnosis or WNV infection is not carried out in Vojvodina. The incidence of clinically manifested human cases is not known. Nowadays, for majority of health professionals in Serbia WNV is the only one in a long line of ARBO viruses transmitted by mosquitoes in remote tropical regions. According to the results of this and previous studies the virus is present in Vojvodina and circulates among urban mosquitoes and humans. The introduction of diagnosis of WNV infection in regular medical practice would contribute to faster establishment of accurate diagnosis and consequently more efficient treatment of serious clinical presentations of WNV infection. Both CFAV and WNV detections in mosquitoes are the first records of such type in Serbia.

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Abbreviations

WNV West Nile Virus
 CFAV cell fusing agent virus
 ELISA enzyme immuno test
 RT PCR real time polymerase chain reaction

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