

# West Nile Virus Outbreak in Humans in Croatia, 2012

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## ABSTRACT

During August and September 2012, seven cases of West Nile neuroinvasive disease were identified in three north-eastern counties of Croatia. Four cases were reported in Osijek-Baranja County, two in Brod-Posavina County and one in Vukovar-Srijem County. The median age of the patients was 62.7 years. All patients were hospitalized for 2–5 weeks. The patients from Slavonski Brod had more severe clinical presentation of disease with prolonged hospitalization. Medical entomological research was carried out in 64 localities, where 1785 mosquitoes were captured. Among the analyzed mosquitoes, 114 were determined to be *Culex pipiens* and subjected to molecular characterization for the presence of virus. No viral RNA was detected in mosquitoes. Subsequent public health measures taken include mosquito control in all settlements where disease was detected.

**Key words:** outbreak, West Nile neuroinvasive disease, human cases, mosquitoes, Croatia

## Introduction

The spreading of West Nile virus (WNV) has been observed in south-eastern parts of Europe, and human infections have been recorded. Human infections have been reported in Italy, Hungary and Romania<sup>1–3</sup> that are in close proximity to Croatia. The actual incidence of WNV in Europe remains under investigation. However, human cases of West Nile neuroinvasive disease (WNND), which can be fatal, have been reported. Nevertheless, a majority of infections are not likely detected since it is estimated that up to 80% of WNV human infections are asymptomatic. In Croatia, past serological studies for WNV infection revealed a seroprevalence of 3% in inhabitants of several Croatian islands<sup>4</sup>. During 2010, WNV was found in horses in nearly all of the lowland counties of Croatia, as well as in Istria County<sup>5</sup>. It is now evident that the virus has spread in the lowland parts of Croatia. While birds are the main reservoir, potential vectors in Croatia are *Culex pipiens* (*Cx. pipiens*) complex, *Aedes vexans* (*Ae. vexans*), *Ochlerotatus caspius* (*Oc. caspius*), *Ochlerotatus cantans* (*Oc. cantans*), *Anopheles maculipennis* and *Aedes cinereus* (*Ae. cinereus*). According to ex-

isting literature<sup>6</sup>, *Cx. pipiens pipiens* var. *molestus* is the most capable vector with a high vector capacity, because feeding of this species includes birds, humans and horses. Although 2012 was a considerably dry year with a lower total number of mosquitoes in the Slavonia region (northeast Croatia), environmental conditions still facilitated spread of WNV, subsequently leading to human infection.

Following an outbreak of WNV in Greece<sup>7</sup> and Romania in 2010<sup>8</sup>, and especially due to recent outbreak in Serbia in 2012, surveillance for WNND was enhanced in Croatia. All health authorities were asked to increase their vigilance. In addition, a history of travel to Serbia was added as part of the case definition for WNND.

## Materials and Methods

### *Detection of specific antibodies against West Nile virus in patients*

Serum samples of seven patients were tested for the presence of IgM and IgG antibodies in National Refer-

ence Laboratory for Arboviruses and Rickettsioses, Croatian Public Health Institute, Zagreb. Laboratory diagnosis was confirmed using a plaque reduction neutralization test (PRNT) at the OIE Reference Laboratory for West Nile Virus at the Istituto G. Caporale (Teramo, Italy).

### Mosquito sampling

Sampling of mosquitoes in 64 localities in three eastern counties of Croatia was done using CDC traps baited with dry ice. Mosquitoes were collected from the 10<sup>th</sup> to 25<sup>th</sup> of September. Traps operated 12 hours, starting in late afternoon. The sampling of mosquitoes in the city of Osijek was carried out under the regular mosquito monitoring program. A total of 114 *Cx. pipiens* mosquitoes were stored in a deep freezer at -80 °C until further analysis.

### West Nile virus detection in mosquitoes

Mosquitoes were pooled according to place of origin and titrated in Minimum Essential Medium. Total RNA was isolated from a 200 µL aliquot of the tissue homogenate after addition of 800 µL TriPure. After transcription into cDNA, semi-nested pan-Flavivirus PCR [8C] was used to amplify viral RNA.

## Outbreak Case Description

On the 4th August 2012, the first suspected case was hospitalized in Slavonski Brod General Hospital. Medical history showed that the patient had travelled recently to Serbia before the onset of disease, indicating that the infection was most likely acquired in Serbia and, thus, may be considered an imported case. The third patient declared that the infection has established due to incidental contact with bird faeces and eye conjunctiva, but mosquito bite also cannot be excluded. Both cases were from Brod-Posavina County, where mosquito abundance was extremely low. In Osijek-Baranja and Vukovar-Srijem County, five patients (Table 1, case 2, 4, 5, and 7) were hospitalised in Osijek Universtiy hospital. The patient declared mosquito bites, but not tick bites. All the patients were treated symptomatically. The patients from

Slavonski Brod (case 1 and 3) had more severe clinical presentation of disease with prolonged hospitalization (45 and 34 days, respectively). Furthermore, they developed neurologic sequels, which required further treatment. The patients from Osijek University Hospital had moderate clinical presentation of disease and were released after 2 to 3 weeks without obvious chronic sequels at the time of this analysis.

The first confirmed WNND case appeared on the 4<sup>th</sup> August 2012 and the last one on 15<sup>th</sup> September 2012. All of them had high IgM ranged from 1.7–4.1 (POZ if >1.1), IgG ranged from 40–132 (POZ if >22 RU/mL). Cases 5 and 7 had negative IgG because serum was taken in the beginning of hospitalization. The sex ratio (male: female) was 3:4. The median age was 62.7 years (age range: 48–77 years). Most cases (n=3) occurred in the 60–69 year old age group (Table 1). Out of 7 cases, two of them lived in urban settings and 5 in rural areas, giving an urban: rural ratio of 2:5.

Neurological symptoms in 5 patients from Osijek hospital were: disturbance of consciousness (apathy, somnolent), restlessness, irritability, hallucinations, general tremors and hyperesthesia. Body temperature rose in all patients between day 3–6, ranging from 38–38.5 °C. Other symptoms included headache, nausea, vomit and photophobia. Cerebrospinal fluid (CSF) was clear, containing 10–517 leucocytes (lymphocytes 15–75%, monocytes 3–23%, neutrophils 2–82%). Patient EEGs were diffusely dysrhythmic or sideways dysrhythmic.

Two patients from Slavonski Brod (cases 1 and 3 in Table 1) had more severe course of disease of WNND, and full report of these cases can be found below.

The first patient was a previously healthy 65 year old male. On August 12th, he was admitted to the hospital after a persistent 7-day fever and headache. He complained of malaise, weakness, nausea and lack of appetite. Upon admission, he was febrile with a temperature of 38 °C and a pulse rate of 98 beats/min, blood pressure of 130/80 mm Hg and respiratory rate of 18 breaths/min. He presented as a mildly ill patient. He was alert and oriented. There were no meningeal signs present and the physical examination was unremarkable. His leukocyte count was 14.30x10<sup>9</sup>/L with 84% neutrophils, 11% lym-

TABLE 1  
CHARACTERISTICS OF PATIENTS WITH WNND IN CROATIA, 2012.

Case	Date of hospitalization*	Age	Sex	Place	County	Day of taking the serum
1.	4. Aug. (7)	65	M	Slavonski Brod	Brod-Posavina	30.
2.	20. Aug. (4)	48	F	Budimci	Osijek-Baranja	21.
3.	25. Aug. (6)	60	F	Slavonski Brod	Brod-Posavina	19.
4.	26. Aug. (3)	77	F	Vukovar	Vukovar-Srijem	15.
5.	4. Sep. (3)	76	M	Belišće	Osijek-Baranja	6.
6.	12. Sep. (5)	48	F	Kuševac	Osijek-Baranja	13.
7.	15. Sep. (6)	65	M	Petrijevci	Osijek-Baranja	8.

\* Numbers in parentheses in second colon represent days with the symptoms of disease before hospitalization

phocytes and 4% monocytes. Lab reports indicated  $4.89 \times 10^{12}/L$  red blood cells and  $187 \times 10^9/L$  platelets count. Serum transaminases were slightly elevated: AST 43 U/L (normal range 11–38 U/L) and ALT 65 U/L (normal range 12–48 U/L). CRP was 18.7 mg/L (normal range <5 mg/L). Other results of serum laboratory analysis were within normal ranges. Lumbar puncture (LP) was performed and his cerebrospinal fluid (CSF) was clear, containing 380 leucocytes and 160 erythrocytes per  $\text{mm}^3$ . CSF glucose level was 2.9 mmol/L and protein level was 1.81 g/L (normal range 0.17–0.37 g/L). No abnormalities were seen on the brain CT scan. On the fourth day of his hospitalization, he became somnolent and dysarthric. Furthermore, flaccid paraparesis occurred. The next day, paraparesis deteriorated to lower limb flaccid paralysis, and right arm flaccid paralysis also occurred. His EEG was diffusely dysrhythmic with slow theta rhythm. Electromyography detected mixed motor polyneuropathy, particularly in lower limbs. Then he became afebrile but, after one week, he developed pneumonia and was febrile again. Although improvement was slow, he was discharged from the hospital and referred to a physical therapy institution.

Case 3 was a 60 year old female with a previous history of mild hypertension. She was admitted to the hospital on the 6th day of her illness and presented with nausea, vomiting and left arm tremor. On the 5th day of her illness, she became febrile and complained of headache, myalgias and decreased appetite. During hospital admission, she was febrile with a temperature of 38.5 °C, and blood pressure was 160/85 mm Hg with a pulse rate of 94 beats/min. She was tachypneic with a respiratory rate of 30 breaths/min and blood oxygen saturation of 94%. Her physical examination was normal with no neck rigidity. Laboratory tests showed that leucocytes count was  $9.94 \times 10^9/L$  with 77% neutrophils, 14% lymphocytes and 9% monocytes. CRP was 4.6 mg/L (normal range <5 g/L). LP was done and her CSF was clear. CSF protein level was 0.96 g/L (normal range 0.17–0.37 g/L). On the second day of hospitalization, flaccid paralysis of her left arm occurred and, at the same time, she became confused. Brain CT scan was normal and EEG was diffusely dysrhythmic with slow theta rhythm. The next day, peripheral facial nerve paralysis occurred. From admission, she was treated with symptomatic therapy only. Over the next week, her consciousness slowly improved and she

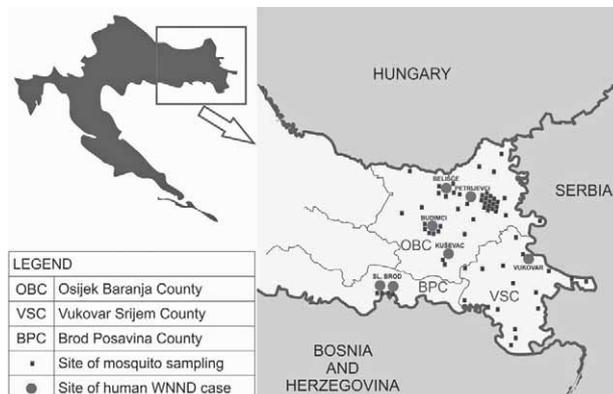


Fig. 1. Distribution of WNV human cases and places of mosquito sampling in Croatia in 2012.

became afebrile, so physical therapy was started. After 28 days of hospitalization, she was discharged and referred to a physical therapy institution.

#### Medical entomological study

Immediately after the first human case of WNV, sampling of mosquitoes was organized. A total of 1785 mosquitoes were caught. The number of sampling places differed from county to county. In Osijek-Baranja County, Vukovar-Srijem County and Brod-Posavina County, there were 43, 17 and 4 sampling sites, respectively (Figure 1). In villages where WNV cases originated (Budimci and Belišće), 4 CDC traps were set up, and one of those being in the backyard of one WNV patient. *Ae. vexans* comprised of 1634 specimens (91.54%), while 114 specimens (6.39%) were determined to be *Cx. pipiens* complex. Species distribution was normal for this part of the year (Table 2). All *Cx. pipiens* complex specimens tested using pan-Flavivirus PCR were negative for WNV.

#### Public health measures

Surveillance in Croatia had been gradually increased following reports of the outbreak of WNV in Serbia and subsequent confirmation of the first case of human infection in Croatia. The Ministry of Health and public health authorities organized several meetings and recommended mosquito control measures. The Public Health Institute of Osijek-Baranja County organized mosquito con-

TABLE 2  
NUMBER OF MOSQUITOES SAMPLED IN DIFFERENT COUNTIES DURING THE WNV OUTBREAK IN CROATIA DURING 2012.

	Osijek-Baranja County	Osijek	Vukovar-Srijem County	Brod-Posavina County
<i>Aedes vexans</i>	816	789	26	3
<i>Culex pipiens</i>	48	39	26	1
<i>Ochlerotatus caspius</i>	16	5	–	–
<i>Anopheles maculipennis c.</i>	5	5	2	–
<i>Anopheles hyrcanus</i>	2	1	1	–
TOTAL	887	839	55	4

trol. Larvicide treatment (based on *Bti*) of 2108 small water bodies in villages (Budimci, Belišće, Vukovar and Kuševac) with suspected human cases was carried out door-to-door from the 18<sup>th</sup> to 29<sup>th</sup> of September 2012. Adulticiding was carried out twice over three days (from the 20<sup>th</sup> to 28<sup>th</sup> of September) at five localities in total of 2900 ha using the ULV method for aerial spraying.

## Discussion

The current report described the first documented WNV outbreak and detection of acute WNV infection in humans in Croatia. Previously, WNV had been detected only in horses in eastern Croatia with a seroprevalence as high as 3.5%<sup>5,9</sup>. Ongoing research in 2011 and 2012 confirmed the presence of WNV in horses with an even higher seroprevalence than in 2010 (unpublished data), meaning that the virus has been present in Croatia for quite some time. In such circumstances, human infection was bound to occur. A connection between illness in horses and humans was established in Andalusia in 2010<sup>10</sup>, indicating that it would be beneficial to investigate the predictive potential of this relationship. Indeed, disease outbreak in horses may be a precursor for the occurrence of the same disease in humans.

Two of seven Croatian patients had a severe form of WNND with chronic neurologic sequels. Fortunately, no fatalities were reported during the outbreak in Croatia. Further follow up and clinical examination of all seven patients is in process. Consistent with cases from other countries in Europe<sup>1–3</sup>, WNND afflicted elderly people in Croatia with a median age of 62.7 years. Specifically, five of seven patients were over 60 years of age. Similar to previous outbreaks in this part of Europe, WNV infection in humans in Croatia occurred during the late summer (August–September)<sup>1–3</sup>.

Over the last three decades, cases of WNV infection have been reported in humans and horses in the Mediterranean Basin. Most of them were determined to be strains of the Lineage 1 included in the European Mediterranean/Kenyan cluster<sup>11</sup>. In recent years, however, the Israeli/American cluster of Lineage 1 and Lineage 2 of WNV have been reported in countries near Croatia, such as Hungary and Austria<sup>11–13</sup>. A WNV Lineage 2 strain was also detected in birds and mosquitoes in Greece during a 2010 WNV outbreak in humans. Phylogenetic analysis revealed high sequence similarity (>99%) with other WNV Lineage 2 strains in Austria and Hungary (2004–2009)<sup>14</sup>. The whole genome characterisation of that strain showed that it presented genetic relationship to the Lineage 2 Hungarian strain, it was suggested that an amino acid substitution H249P in the NS3 protein might play a role in the increased pathogenicity of the Greek strain<sup>15</sup>. A recent study in Serbia showed that 12% of horses had specific antibodies against WNV<sup>16</sup>, followed by an outbreak with severe and fatal human cases in 2012<sup>17</sup>. In 2012, the first human case of WNV was confirmed in Kosovo<sup>18</sup>.

Over the last few years, a significant public health concern of WNV infection in humans and, especially, blood donors has been raised in Italy. In summer of 2012, 13 confirmed human cases of WNV were diagnosed in northern Italy, including patients with neuroinvasive disease. Five WNV-positive blood donors were confirmed as well<sup>19,20</sup>. WNV infection has become an important topic in relation to blood, tissue and organ safety following several reports of contaminated blood supplies and organs during the WNV outbreak in the United States<sup>21,22</sup>. Based on data from epidemiological surveillance of horses and wild birds, as well as reports of human WNV neuroinvasive infections, blood safety measures in Italy were established. Specifically, WNV RNA screening by nucleic acid amplification testing (NAT) of all blood donations from donors living in the WNV endemic was introduced<sup>23</sup>. Despite implementation of NAT screening of blood donors for WNV, several »breakthrough« WNV transfusion transmission cases were reported between 2004–2008, suggesting that current plasma-based assays are unable to detect all WNV-infectious donations and that additional molecular tests may be required<sup>24</sup>. As the first acute cases of WNV in humans in Croatia were reported in 2012, there is no sufficient and relevant epidemiological data on the spread of WNV among humans, horses and birds. Furthermore, no compulsory testing for WNV in blood donors has been introduced. However, blood donors are temporarily rejected 28 days after leaving in a WNV endemic area.

Out of several potential vectors for WNV in Croatia, the likeliest vector is *Cx. pipiens* complex, because other above mentioned species have low vector capacity. For this reason, only *Cx. pipiens* complex specimens were included in the analysis and tested for the presence of WNV. In the fauna of Osijek, this species accounts for 5.86% of the mosquito population measured over prolonged period<sup>25</sup>, which is consistent with results of the current study (6.39%). In the collected *Cx. pipiens* complex mosquitoes, no viral WNV RNA was detected. The small number of tested mosquitoes limits the current study<sup>26</sup>. As the summer of 2012 was rather dry, conditions for mosquito breeding were unfavourable, resulting in a lower total number of mosquitoes. Nevertheless, other conditions must have influenced and facilitated an efficient transfer of WNV to humans. Probably high rate of infected birds, small population of mosquitoes in close proximity to humans, resulted in human outbreak. Further extensive investigations of WNV spreading in humans, birds and horses are necessary to be done in Croatia.

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## POJAVA BOLESTI KOD LJUDI IZAZVANE VIRUSOM ZAPADNOG NILA U HRVATSKOJ TIJEKOM 2012.

### SAŽETAK

Tijekom kolovoza i rujna 2012. godine sedam ljudi je oboljelo od neuroinvasivnih bolesti izazvane virusom Zapadnog Nila u tri sjeveroistočne županije u Hrvatskoj. Oboljela su četiri bolesnika u Osječko-baranjskoj, dva u Brodsko-posavskoj i jedan u Vukovarsko-srijemskoj županiji. Prosječna starost oboljelih je bila 62,7 godina. Svi pacijenti su hospitalizirani od 2 do 5 tjedana. Pacijenti koji su hospitalizirani u bolnici u Slavonskom Brodu imali su teže kliničke simptome te su duže hospitalizirani. Medicinsko-entomološka istraživanja obavljena su na ukupno 64 postaje u sve tri istraživanje županije. Od ukupno uhvaćenih 1.785 komaraca 144 ih je determinirano kao *Cx. pipiens*, tj potencijalni prijenosnik virusa Zapadnog Nila. Molekularnom analizom u tim jedinkama nije pronađena virusna RNA. U kontekstu sprječavanja širenja epidemije organizirana je protuepidemijska dezinfekcija koja se sastojala od larvicidnog i adulticidnog tretmana u mjestima gdje je zabilježena bolest.