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Vilibić Čavlek, Tatjana; Savić, Vladimir; Sabadi, Dario; Perić, Ljiljana; Barbić, Ljubo; Klobučar, Ana; Miklaušić, Božana; Tabain, Irena; Santini, Marija; Vucelja, Marko; ...

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Running title: West Nile and Usutu infections in Croatia

Tatjana Vilibic-Cavlek[1,2]*, Vladimir Savic[3]*, Dario Sabadi[4,5], Ljiljana Peric[4,5], Ljubo Barbic[6], Ana Klobucar[7], Bozana Miklausic[8], Irena Tabain[1], Marija Santini[2,8], Marko Vucelja[9], Elizabeta Dvorski[10], Tamara Butigan[10], Gordana Kolaric-Sviben[11], Tanja Potocnik-Hunjadi[12], Mirta Balenovic[3], Maja Bogdanic[1], Zdravko Andric[13], Vladimir Stevanovic[6], Krunoslav Capak[1], Marina Balicevic[14], Eddy Listes[15], Giovanni Savini[16]

*Authors with equal contribution

[1]Croatian Institute of Public Health, Zagreb, Croatia

[2]School of Medicine, University of Zagreb, Zagreb, Croatia

[3]Croatian Veterinary Institute, Zagreb, Croatia

[4]Clinical Hospital Center Osijek, Osijek, Croatia

[5]Medical Faculty, Josip Juraj Stossmayer University of Osijek, Osijek, Croatia

[6]Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia

[7]Andrija Stampar Teaching Institute of Public Health, Zagreb, Croatia

[8]University Hospital for Infectious Diseases "Dr Fran Mihaljevic", Zagreb, Croatia

[9]Faculty of Forestry, University of Zagreb, Zagreb, Croatia

[10]General Hospital Varazdin, Varazdin, Croatia

[11]General Hospital "Dr Tomislav Bardek", Koprivnica, Croatia

[12]County Hospital Cakovec, Cakovec, Croatia

[13]General County Hospital Pozega, Pozega, Croatia

[14]General Hospital "Dr Josip Bencevic", Slavonski Brod, Croatia

[15]Croatian Veterinary Institute, Regional Institute Split, Split, Croatia

[16]OIE Reference Center for West Nile Disease, Istituto Zooprofilattico Sperimentale "G. Caporale", Teramo, Italy

Corresponding author

Tatjana Vilibic-Cavlek, MD, PhD

Department of Virology, Croatian Institute of Public Health

Rockefellerova 12, 10000 Zagreb, Croatia

e-mail: tatjana.vilibic-cavlek@hzjz.hr

Summary

In 2018, Croatia reported the largest outbreak of West Nile virus (WNV) infections as well as the re-occurrence of human Usutu virus (USUV) infections. For the first time, fatal WNV and USUV infections were detected in wild birds. We analyzed epidemiological characteristics and molecular epidemiology of WNV and USUV infections detected during 2018 transmission season. From April to November, 178 patients with neuroinvasive disease and 68 patients with febrile disease were tested for WNV and USUV. Viral RNA was detected in cerebrospinal fluid (CSF) and urine samples using a real-time RT-PCR. Positive samples were tested by nested RT-PCR and nucleotide sequencing. IgM/IgG antibodies were detected in serum/CSF samples using ELISA with confirmation of cross-reactive samples by virus neutralization test (VNT). WNV neuroinvasive disease was confirmed in 54 and WNV fever in 7 patients from 10 continental Croatian counties. Areas affected in 2018 were those in which cases occurred in previous seasons, while in three areas human cases were reported for the first time. Phylogenetic analysis of six strains from patients residing in different geographic areas showed circulation of WNV lineage 2. In three patients, neuroinvasive USUV infection was confirmed by RT-PCR or VNT. Sequence analysis of one detected strain revealed USUV Europe 2 lineage. During the same period, a total of 2,574 horse and 1,069 poultry serum samples were tested for WNV antibodies using ELISA. Acute asymptomatic WNV infection (IgM antibodies) was documented in 20/0.7% horses. WNV IgG antibodies were found in 307/11.9% horses and 125/12.7% poultry. WNV RNA was detected in two goshawks and USUV RNA in one blackbird from northwestern Croatia. In the Zagreb area, 3,670 female mosquitoes were collected. One *Culex pipiens* pool collected in July tested positive for USUV RNA. Our results highlight the importance of continuous multidisciplinary "One health" surveillance of these emerging arboviruses.

Key words: West Nile virus, Usutu virus, prevalence, molecular epidemiology, climate, Croatia

Introduction

West Nile virus (WNV) is one of the most widely distributed arboviruses. In nature, virus is maintained in an enzootic cycle between birds and mosquitoes. Numerous bird species are competent amplifier hosts for WNV, while mosquitoes of the genus *Culex* are the main vectors. However, members of the family *Corvidae* (crows, jays and magpies) are susceptible to infection and may develop severe illness with a high mortality rate (Gamino & Hofle, 2013). Due to a low-level viremia, humans and horses represent accidental dead-end hosts for WNV (Petersen et al., 2013). Although majority of human infections are asymptomatic, some infected persons (~20%) develop non-specific febrile disease (WNV fever) and less than 1% develop WNV neuroinvasive disease with a fatality rate of around 10% (Hart et al., 2014). In addition, approximately 10% of horses infected with WNV presented neurological disorders (Castillo-Olivares & Wood, 2004). Phylogenetic studies have revealed the existence of multiple WNV genetic lineages, of which lineage 1 and 2 circulate in Europe and represent the most important human pathogens (Rizzoli et al., 2015). In recent years, WNV epidemiology has changed in many European countries where the occurrence of outbreaks has dramatically increased in both humans and horses (Tran et al., 2017; Kolodzijek et al., 2018). In Croatia, first human cases of WNV neuroinvasive disease were reported in 2012 (Pem-Novosel et al., 2013), thereafter small outbreaks (2013, 2017) and sporadic cases were continuously notified in continental Croatian counties (Vilibic-Cavlek et. al, 2014; unpublished data of the Reference Center for Diagnosis and Surveillance of Viral Zoonoses Croatian Ministry of Health, Croatian Institute of Public Health). In addition to human cases, asymptomatic WNV infections and seropositivity in sentinel horses (Barbić et al., 2012), as well as in poultry were detected in the same geographic areas. However, there are very few data on the genetic characterization of WNV strains detected in Croatia (Kurolt et al., 2014).

Usutu virus (USUV) is a mosquito-borne arbovirus, closely related to WNV. The natural cycle of USUV is similar to WNV involving birds as the main amplifying reservoir hosts and *Culex* mosquitoes as main vectors (Gaibani & Rossini, 2017). In Europe, USUV emerged in 1996,

causing considerable die-off of Eurasian blackbirds (Weissenbock et al., 2013). In the following years, USUV-associated bird mortality, mainly among blackbirds and great gray owls was documented in many European countries (Bakonyi et al., 2017). In the summer of 2016, Belgium, France, Germany and the Netherlands reported widespread USUV activity in wild and captive birds (Rijks et al., 2016; Cadar et al., 2017). However, human USUV infections are rarely reported. Like WNV, USUV infections in humans are usually asymptomatic while neuroinvasive disease is rare, mainly in immunocompromised patients (Pecorari et al., 2009; Cavrini et al., 2009). Sporadic cases of USUV infections with atypical neurological presentations are also reported (Simonin et al., 2018). However, a recently published study from north-western Italy showed higher seroprevalence of USUV (0.78%) compared to WNV (0.42%) indicating that USUV circulation was more abundant than that of WNV (Faggioni et al., 2018). There are at least six genetic lineages of USUV which are distinct in two major groups, African and European (Engel et al., 2016; Cadar et al., 2017).

In Croatia, USUV seropositive horses were detected in 2011 in northwestern counties (Barbic et al., 2013). During the 2013 WNV outbreak, first three human cases of neuroinvasive USUV infection were reported in Zagreb and its surroundings (Vilibic-Cavlek et al., 2014; Santini et al., 2015). USUV positive mosquitoes were detected for the first time in 2016 (Klobučar et al., 2018), while positive birds were not documented so far. In addition, there are no data on the genetic characterization of USUV.

In 2018, Croatia experienced the largest outbreak with more than 60 confirmed human cases of WNV neuroinvasive disease and WNV fever. Re-occurrence of human USUV neuroinvasive infections was also reported. In addition, fatal WNV and USUV infection in wild birds were detected for the first time. We analyzed epidemiological characteristics and molecular epidemiology of WNV and USUV infections detected in Croatia during 2018 transmission season.

Material and Methods

Testing of human samples

From April to November 2018, a total of 178 patients with neuroinvasive disease and 68 patients with symptoms compatible with WNV fever were tested for the presence of WNV and USUV at the National Reference Laboratory for Arboviruses, Croatian Institute of Public Health. Viral RNA was extracted from cerebrospinal fluid (CSF), urine and/or serum samples using a High Pure Viral Nucleic Acid Kit (Roche Applied Science, Mannheim, Germany). TaqMan real-time RT-PCR assays for detection of WNV and USUV RNA were performed according to the protocols of Tang et al. (2006) and Nikolay et al. (2014), respectively, using a Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA) and Rotor-Gene Q real-time PCR cycler (Hilden, Germany). Samples identified as positive using the real-time RT-PCR assays were subjected to conventional RT-PCR using PrimeScript™ One Step RT-PCR Kit Ver.2 (Takara Bio Inc, Kusatsu, Japan) and panflavi primers targeting the NS5 gene (FP: 5'-TACAACATGATGGGVAARAGAGAGA-3', RP: 5'-AGCATGTCTTCYGTBGTCATCCAYT-3') to amplify 1085 bp (WNV) and 1084 bp (USUV) products according to the protocol of Weissenböck et al. (2002). For samples that yielded faint PCR product, nested PCR using EmeraldAmp MAX PCR Master Mix (Takara Bio Inc) and WNV internal primers (FP-WNV: 5'-AGAGAGAAGAAGCCTGGAGAG-3', RP-WNV: 5'-CTTTGGTGATGCGTGTGTC-3') amplifying 263 bp product and for USUV internal primers (FP-USUV: 5'-TTCTGGAGGAGGTGTTGAAGG-3', RP-USUV: 5'-CACTCTGGCACGTCTTTCTG-3') amplifying 585 bp product was performed. All nested PCR reactions were performed under the same PCR conditions as for the RT-PCR, but with exclusion of the RT step. Conventional PCR was carried out in Biometra T3000 PCR Cycler (Biometra, GmbH, Göttingen, Germany). Amplified products were visualized on 1% agarose gel. DNA samples extracted from excised gel fragments were Sanger sequenced in both directions by Humanizing Genomics, Macrogen Inc. (Amsterdam, The Netherlands) with use of the internal primers. After sequencing, the raw nucleotide sequences were assembled and the primer sequences were trimmed off. Genotyping and phylogenetic grouping of obtained

sequences were based on comparison with strains retrieved from the GenBank and obtained using BLAST algorithm (http://www.ncbi.nlm.nih.gov). Maximum likelihood phylogenetic analysis was conducted and the evolutionary analyses performed by using MEGA7 (Kumar et al., 2016).

In addition, human CSF and serum samples were tested for WNV IgM and IgG antibodies, WNV IgG avidity and USUV IgG antibodies. For 34 patients, acute and convalescent serum samples were obtained. Serologic tests were performed using commercial enzyme-linked immunosorbent assays (ELISA; Euroimmun, Lübeck, Germany). Results of the ELISA were interpreted as follows: IgM ratio <0.8 negative, 0.8-1.1 borderline, $\geq$1.1 positive; IgG relative units (RU/ml) <16 negative, 16-22 borderline, $\geq$16 positive. WNV IgG positive samples were tested for IgG avidity using urea as a denaturing agent. The IgG avidity index (AI) was calculated and expressed as percentage using the extinction values with and without urea treatment and interpreted as follows: <40% low AI, 40%-60% borderline AI, >60% high AI (Vilibic-Cavlek et al., 2018).

Cross-reactive samples were confirmed using a virus neutralization test (VNT) at the OIE Reference Center for West Nile Disease, Istituto Zooprofillatico Sperimentale "G. Caporale", Teramo, Italy. The antibody titer was defined as the reciprocal value of the highest serum dilution that showed 100% neutralization. Titers of $\geq$10 were considered positive. Prior to VNT, WNV and USUV antigens (strains Eg-101 and 939/01, respectively) were titrated by 50% TCID ($TCID_{50}$) using Vero cells. After four days, the titer was determined using the Reed and Muench formula (Di Gennaro et al., 2014).

In order to analyze the epidemiological characteristics of WNV and USUV infections, demographic (age, gender, occupation) and epidemiological data (date of disease onset, place of residence, traveling to areas with documented virus circulation, mosquito bites) were collected using a questionnaire. In addition, climatic and meteorological data were obtained from the Croatian Meteorological and Hydrological Service (CMHS, 2018). Correlation analysis (Pearson correlation coefficient; R) was used to evaluate the relationship between the number of WNV cases and the average monthly air temperatures (°C) along with total

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precipitation (mm) in two areas with the largest number of reported cases (eastern; E and north-western; NW counties).

Testing of horse samples

During the same time period, a total of 2,574 horse serum samples were collected and tested for WNV IgM and/or IgG antibodies using a blocking ELISA (Ingezim West Nile COMPAC, Madrid, Spain). For each sample, inhibition percentage (IP) was calculated according to the formula: IP=100-[(sample OD/negative control OD) x 100]. Samples with IP $\leq$30% were considered negative, 30-40% equivocal and $\geq$40% positive. IgM positive horses were additionally tested for the presence of WNV RNA (as described above). The horses included in the study had no vaccination history and were asymptomatic at the time of sampling.

Testing of poultry samples

A total of 1,069 poultry (chickens, turkeys) were collected and tested for the presence of WNV IgG antibodies using a blocking ELISA (Ingezim West Nile COMPAC, Madrid, Spain). The poultry included in the study were older than three weeks (4-36 weeks) and were hatched in 2018. This selection assured reliable demonstration of the viral activity in the current year and exclusion of maternally derived antibodies as well as WNV antibodies from previous seasons.

Testing of bird samples

Within a flavivirus surveillance program introduced in Croatia in 2013, passive surveillance of WNV and USUV was conducted. Supernatant fluids of brain tissue samples homogenized in PBS (1:1) of 35 dead wild birds from families Passeridae (25), Accipitridae (4), Laridae (3), Anatidae (1), Ciconiidae (1) and Turdidae (1) were tested for presence of WNV and USUV RNA (as described above).

Testing of mosquito samples

WNV and USUV screening in mosquitoes was also performed in northwestern Croatia. Adult mosquitoes were collected in the intervals of two weeks from May to November 2018 in Zagreb area at 34 locations in different habitats in green spaces, urban and suburban settlement. Sampling was performed using BG-Sentinel traps, CDC traps with dry ice and human landing collection with aspirator. Traps were exposed in the late afternoon and collected early in the morning. The female mosquitoes were identified morphologically (Becker et al., 2010) immediately after sampling and grouped with a maximum number of 40 individuals per pool on the basis of the date of collection, location, and species and tested for the presence of WNV and USUV RNA (as described above). Pooled samples were stored at -70°C until testing.

The study was approved by the Ethics Committees of the Croatian Institute of Public Health and the University Hospital for Infectious Diseases "Dr Fran Mihaljevic", Zagreb.

Results

Human WNV and USUV infections

Fifty-four Croatian patients with neuroinvasive disease and 7 patients with WNV fever met clinical and laboratory criteria for "confirmed cases" of the European Union Case definition for WNV infection (ECDC, 2018). In all patients IgM antibodies were demonstrated with IgG antibodies of low avidity (3-33%) in 59 and borderline avidity (44% and 52%, respectively) in two patients. In 14 patients, WNV IgG seroconversion was documented in paired serum samples. Demographic, clinical and laboratory characteristics of patients with WNV disease are presented in table 1. Patient's age ranged from 6 to 90 (mean 61.1 years) with majority (53/85.5%) aged $\geq$50 years. About half of the patients (31/50.8%) were residents of suburban/rural areas and 38/62.3% patients reported having been exposed to mosquito bites occasionally (28/45.9%) or frequently (10/16.4%). Three additional WNV cases were imported, from Serbia, Italy and Ohio (USA). The first case developed symptoms on 15 July and the last one on 11 October (figure 1). WNV infections occurred in 10 continental Croatian

counties (figure 2). Areas affected in 2018 were those in which cases were also reported between 2012 and 2017 (Pem-Novosel et al., 2013; Vilibic-Cavlek et al., 2014), while in three areas human cases were reported for the first time: Karlovac County (4), Koprivnica-Krizevci (6) and Virovitica-Podravina.County (10) (Figure 2).

WNV RNA was detectable in 48 tested samples from 39 patients: one blood, 17 serum, 12 CSF and 18 urine samples. Nucleotide sequences were obtained for six strains detected in urine samples of patients with WNV neuroinvasive disease. Phylogenetic analysis showed circulation of the WNV lineage 2 (figure 3).

USUV neuroinvasive disease was confirmed in three patients. In two patients aged 25 and 84 years, respectively, IgG seroconversion was demonstrated, confirmed by detection of USUV neutralizing antibodies. In one immunocompromised patient aged 60 years affected by a chronic lymphocytic leukemia with fatal meningoencephalitis, urine sample was positive for USUV RNA. The sequenced strain belongs to USUV Europe 2 lineage (figure 4). Cases occurred in July, August and September (figure 1) in one north-western and two eastern Croatian counties (figure 2).

WNV infections in horses

A total of 307 (11.9%) horse serum samples tested positive for WNV IgG antibodies. In 20 (0.7%) samples WNV IgM antibodies were detected indicating recent WNV infection. No one IgM positive horse showed clinical symptoms of WNV infection. Three samples (0.01%) showed equivocal result for IgM and 59 samples (2.3%) showed equivocal result for IgG antibodies (suspect WNV infection). No one IgM positive animal was WNV RNA positive. The first IgM seropositive horse was detected in March, thereafter IgM positive animals were continuously reported until November (figure 1). Acutely infected animals were recorded in seven counties: Zagreb (21), Medimurje (20), Koprivnica-Krizevci (6), Bjelovar-Bilogora (7), Virovitica-Podravina (10), Brod-Posavina (11) and Vukovar-Srijem (16) (figure 2), while IgG seropositive animals were recorded in all continental counties. IgG seroprevalence rates

varied from 3.2% to 26.0% with the highest seropositivity in regions with the largest number of human cases (eastern; E and north-western; NW counties) (figure 5a).

WNV infections in poultry

Confirmed or probable WNV infection was documented in 125 (12.7%) poultry serum samples. The seroprevalence rates were from 1.7% to 39.5% (figure 5b). Seropositivity correlated with the number of human cases in majority analyzed areas, except four counties. In Osijek-Baranja County (14), lower seroprevalence was detected (6.1%) in contrast to large number of human cases. In three western counties: Krapina-Zagorje (2), Sisak-Moslavina (3) and Bjelovar-Bilogora (7), no human cases were reported while seropositive poultry was detected with seroprevalence rates of 10.1%, 4.3% and 1.7%, respectively.

WNV and USUV infections in wild birds and mosquitoes

In September 2018, a female and a male goshawk (*Accipiter gentilis*) from the same aviary were real-time RT-PCR positive for WNV RNA and one blackbird (*Turdus merula*) was real-time RT-PCR positive for USUV RNA (figure 1). Phylogenetic analysis showed WNV lineage 2 and USUV Europe 2 lineage (figures 3 and 4). Geographic locations of positive birds are presented in figure 2.

During entomologic survey, a total of 3,670 female mosquitoes were collected. Six mosquito species were detected: *Culex pipiens* (1,489/40.6%), *Ochlerotatus sticticus* (1432/39.0%), *Aedes albopictus* (476/13.0%), *Ae. vexans* (189/5.1%), *Oc. rusticus* (71/1.9%) and *Ae. cinereus* (13/0.4%). No one of 177 tested mosquito pools was WNV RNA positive. One pool of *Culex pipiens* mosquitoes trapped on 19 July in Zagreb (figure 2) tested positive for USUV RNA. Detected sequence clustered within USUV Europe 2 lineage (figure 4).

Climate and meteorological conditions in 2018

The Croatian mainland is characterized by continental climate with cold winters and hot summers. The mean annual precipitation is 800-900 mm, with a minimum in winter (~50 mm in February) and a maximum in summer (~100 mm in August).

In two geographic regions with the largest number of reported cases (E and NW counties), climate characteristics were analyzed. Absolute maximum air temperatures in April and May 2018 in both analyzed regions were above the multiannual average (1961-2017). Anomalies were within the range from 4.8-5.2°C in April and 3.5-4.1°C in May. According to percentile ranks and classification ratings, thermal conditions for both months fall under the extremely warm category (99 percentile). In summer and autumn 2018, absolute maximum air temperatures were also above averages (0.7-3.3°C in E and 2.2-4.4°C in NW regions). In October 2018, when the last WNV cases were detected, thermal conditions were extremely warm (99 percentile) in the wider area of Zagreb and very warm (92-96 percentile) in other analyzed regions (CMHS, 2018).

In both E (12, 14, 16) and NW (1, 21) Croatian counties (figure 6), positive moderate correlation (Pearson correlation coefficient; r=0.50 in E; r=0.49 in NW) was determined between the number of WNV cases and average monthly air temperatures (°C). Negative weak to moderate correlation coefficient (r=-0.13 in E; r=-0.56 in NW) was found between the number of WNV cases and total monthly precipitation (mm).

Discussion

Human WNV infections have sharply increased in 2018 as compared to the previous seasons. This is largely due to the unusually early start of the transmission season in Europe, which normally lasts from July to October with the case numbers usually peaking between mid-August and mid-September (Aberle et al., 2018; Burki, 2018; Hausig et al., 2018). The first WNV cases were notified by Greece, with the earliest disease onset on 31 May. In addition, an early start of the transmission season in 2018 was also observed among equids (Hausig et al., 2018). In 2018, as of 13 December 2018, EU Member States and Neighboring countries have reported 2,083 human cases, with 181 deaths due to WNV infection. Moreover, 285 outbreaks among equids have been reported (ECDC, 2018).

In the current season, Croatia recorded the largest outbreak with 54 confirmed autochthonous WNV neuroinvasive disease and 7 WNV fever cases detected in 10

continental counties. In addition, acute asymptomatic infection was documented in 20 horses. Acutely infected animals were detected in the same counties where human cases occurred. In comparison with other European countries, our results reflected a similar seasonality pattern with the first IgM seropositive horses detected in March. Since IgM antibodies in horses are short-lived (Castillo-Olivares et al., 2011), IgM detection suggests the WNV activity in the current season. WNV infections in horses preceded human infections. The first human cases were detected in July with the peak in August (59.0%). In contrast to previous years, WNV transmission season extended to October 2018. Similar to the increase in the number of acute cases, higher IgG seropositivity rates in horses were noted compared to previous transmission seasons (unpublished data of the Faculty of Veterinary Medicine University of Zagreb). The highest seroprevalence rates were recorded in the same areas as human cases (E 13.9-26.0% and NW counties 4.5-11.6%). In addition, high seropositivity was detected in poultry (E 14.7-39.5%, NW counties 7.9-14.5%). In contrast to a large number of human infections, in Osijek-Baranja County seroprevalence in poultry was 6.1%. Unlike in the other counties, in this county only intensive farming egg-laying chickens were tested which are presumably less exposed to mosquitoes resulting in a lower WNV seropositivity. Although passive monitoring of flaviviruses in wild birds (virological testing of dead migratory birds, corvids and birds of prey) was performed in Croatia since 2013, WNV RNA was detected for the first time in two goshawks in 2018 transmission season. In this study, no one of the tested mosquito pool was positive for WNV RNA. Even in the peak of an outbreak, it is hard to find positive mosquitoes. The best method is to sample mosquitoes around the houses with known WNV cases during the time of transmission. Relatively small number of collected mosquitoes may be the possible reason for negative results of WNV presence in mosquito pools.

WNV lineage 2 has been regularly identified as the cause of local outbreaks or sporadic cases of infection throughout eastern and central Europe (Bagnarelli et al., 2011; Kolodziejek et al., 2015; Ravagnan et al., 2015; Barzon et al., 2015; Zehender et al., 2017; Cotar et al., 2018). So far, only two WNV strains were genetically characterized during the 2013 Croatian

outbreak in Zagreb and its surroundings. The nucleotide sequence of the obtained amplicons were 100% identical and belong to the WNV lineage 2 (Kurolt et al., 2014). In 2018 outbreak, six detected strains from patients with neuroinvasive disease residing in different geographic areas showed circulation of WNV lineage 2, as well. Three WNV strains from northwestern Croatia clustered with viruses from Greece and Serbia, while three other strains, one from northern and two from eastern counties, clustered separately with viruses from different European countries. Although a relatively small nucleotide sequence was analyzed, such diversity indicates introduction and circulation of various WNV strains in Croatia and/or extensive evolution of these viruses due to high viral activity in the 2018 transmission season.

Like WNV, an early start of the USUV transmission was also observed in 2018. Austria detected USUV RNA in 18 blood donations of which the first one was positive in June (Aberle et al., 2018). In Croatia, the first case of USUV neuroinvasive infection was recorded in July, and the last one in September. Molecular characterization of USUV RNA detected in a patient with neuroinvasive disease from eastern Croatia has shown that the virus belongs to Europe 2 lineage as it was the case for the USUV detected for the first time in humans, Italy, 2009 (Gaibani et al., 2013). In addition to human cases, USUV infection was confirmed for the first time in one dead blackbird. One *Cx. pipiens* pool also tested positive for USUV RNA during 2018 transmission season. Both detected strains belong to USUV Europe 2 lineage.

Changes in climatic conditions are hypothesized to play a role in the increasing number of WNV outbreaks observed in Europe in recent years. A recently published study from Northern Italy found relationship between maximum temperatures recorded in the 5th and 6th week prior to diagnosis and the incidence of WNV infection (Moirano et al., 2018). In 2018, climate conditions in continental Croatia from April to October were very or extremely warm. During the whole year, absolute air temperatures were above the multiannual average (CMHS, 2018). In two analyzed regions (E and NW), positive correlation (r=0.50 and r=0.49, respectively) was determined between the number of human cases and average monthly air

temperatures (°C). Similarly, a weak positive correlation was demonstrated between the number of cases and temperature in previous transmission seasons (2012-2017) in both E (r=0.22) and NW region (r=0.20). Higher air temperatures influence vector competence by accelerating the virus replication within mosquitoes (extrinsic incubation period) and prolong their breeding season (Conte et al,. 2015). In addition, increased temperatures cause an upsurge in the growth rates of vector populations, decrease the interval between blood meals and increase viral transmission efficiency to birds (Kilpatrick et al., 2008; Paz, 2015). However, observations on the relationship between precipitation and WNV occurrence are contradictory. While some studies found association between precipitation and WNV incidence (Moirano et al., 2018), the others showed that dry weather induces WNV outbreaks (Wang et al., 2010). Our study showed similar results, i.e. negative correlation between total monthly precipitation and the number of human WNV infections. During previous WNV transmission seasons in Croatia, correlation between the number of cases and precipitation was found to be weak negative in E region (r=-0.07) and weak positive in NW region (r=0.09).

In conclusion, detection of WNV and USUV in humans, horses, poultry, wild birds and mosquitoes in Croatia highlights the importance of a continuing integrated human, animal and vector surveillance ("One health") of these emerging zoonoses. Since WNV became endemic in many Croatian counties, it is important that clinicians are reminded to include WNV in the differential diagnosis of aseptic meningitis during the arbovirus transmission season. In addition, detection of USUV in humans emphasizes the need for increased awareness of neuroinvasive USUV infection, especially in immunocompromised patients.

assistance as well as associates of the Vector and Pest Control Unit from Andrija Stampar Teaching Institute of Public Health, Zagreb, who participated in the field and laboratory work with mosquitoes.

Conflict of interest statement

The authors declare no conflicts of interest.

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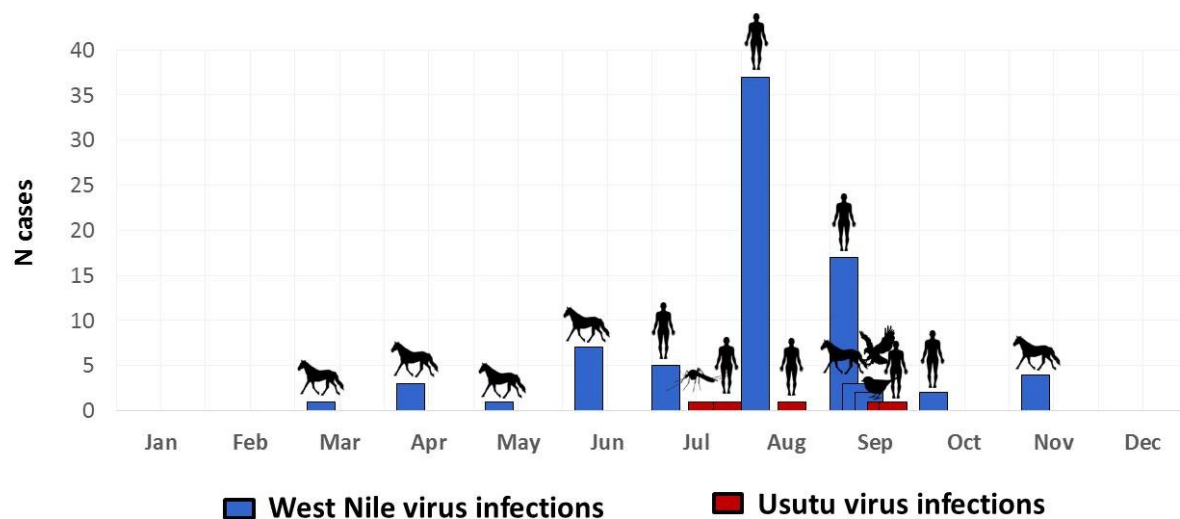
Figures

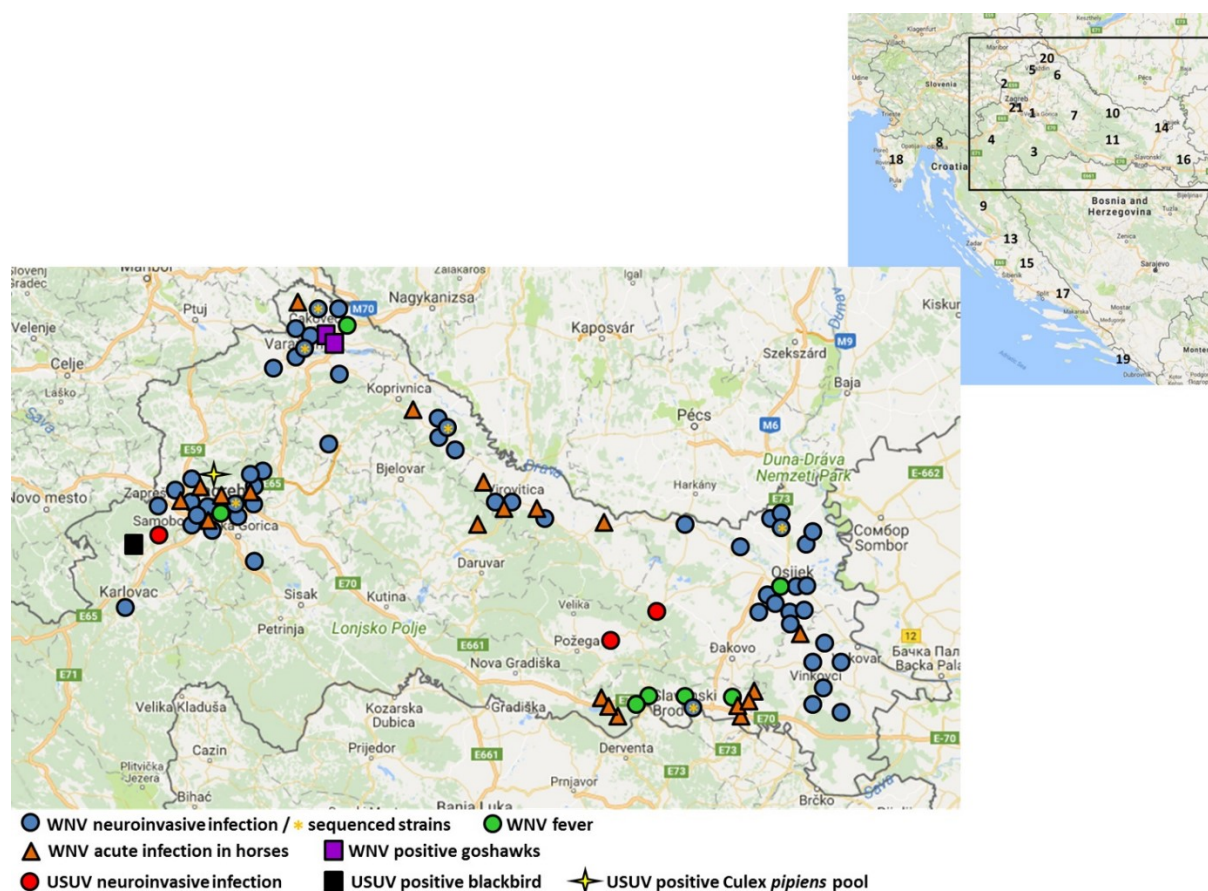Figure 1. Seasonal distribution of West Nile and Usutu virus infections in Croatia, 2018

Figure 2. Geographic distribution of West Nile and Usutu virus infections in Croatia, 2018
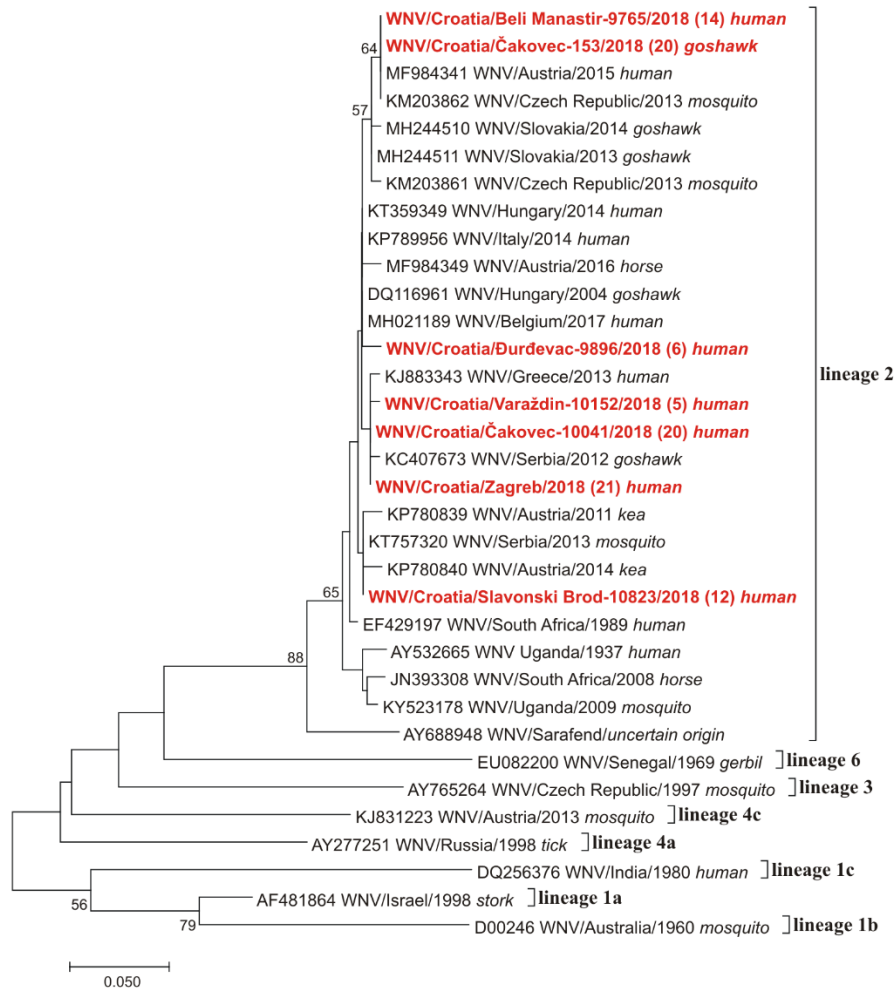
Figure 3. Phylogenetic neighbour-joining analysis of a 222 nucleotide fragment of the WNV NS5 gene (corresponding nucleotide positions 9070-9291 of the B956 strain, GenBank accession number AY532665) detected in Croatia during 2018 and representative WNV strains. GenBank accession numbers, countries of origins, isolation/detection years and sources are indicated at the branches. Viruses from Croatia that were sequenced in this study are marked in bold and red color with county of origin indicated in parentheses. WNV genetic lineages suggested by Rizzoli et al. (2015) are indicated on the right. Lineages 4c, 5 and 7 could not be included in the analysis due to only partial sequence availability. Supporting (≥50%) bootstrap values of 1,000 replicates are displayed at the nodes. Horizontal distances are proportional to genetic distance. Scale bar indicates nucleotide substitutions per site.
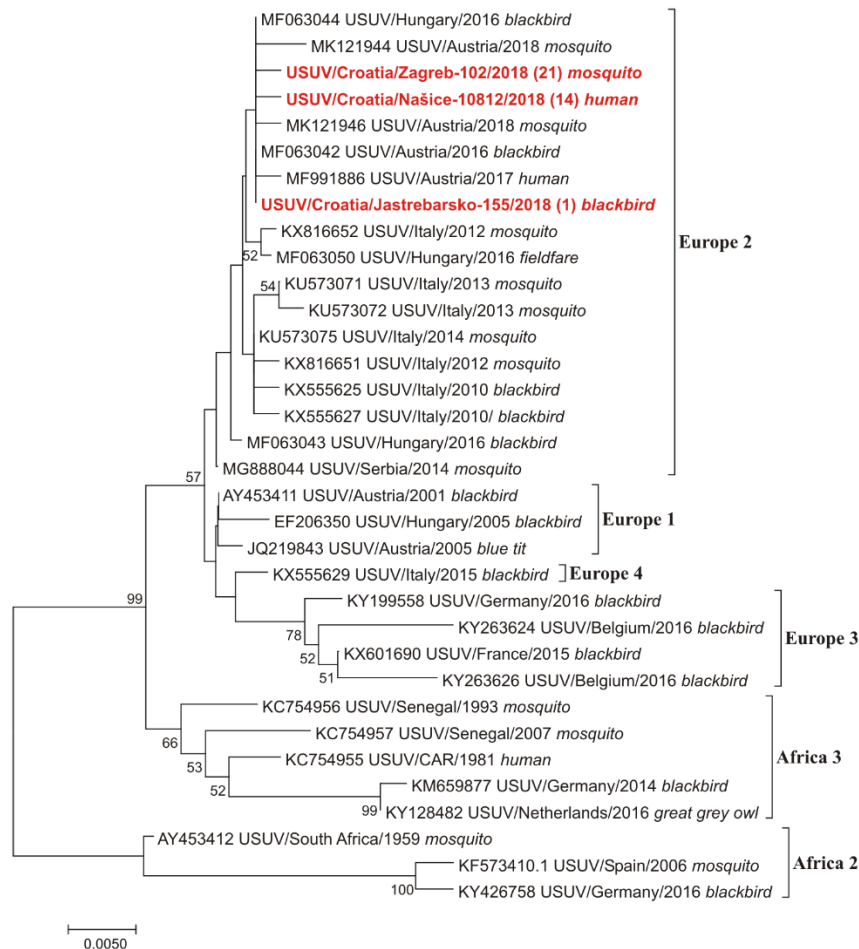
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Figure 4. Phylogenetic neighbour-joining analysis of a 543 nucleotide fragment of the USUV NS5 gene (corresponding nucleotide positions 9758-9216-9758 of the SAAR-1776 strain, GenBank accession number AY453412) detected in Croatia during 2018 and representative USUV strains. GenBank accession numbers, countries of origins, isolation/detection years and sources are indicated at the branches. Viruses from Croatia that were sequenced in this study are marked in bold and red color with county of origin indicated in parenthesis. USUV genetic lineages suggested by Cadar et al. (2017) are indicated on the right. The highly divergent Africa 1 genetic lineage represented by USUV strain from Central African Republic, 1969, (GenBank accession number KC754958) was not included in the analysis in order to increase the resolution of the phylogram. Supporting (≥50%) bootstrap values of 1,000 replicates are displayed at the nodes. Horizontal distances are proportional to genetic distance. Scale bar indicates nucleotide substitutions per site.
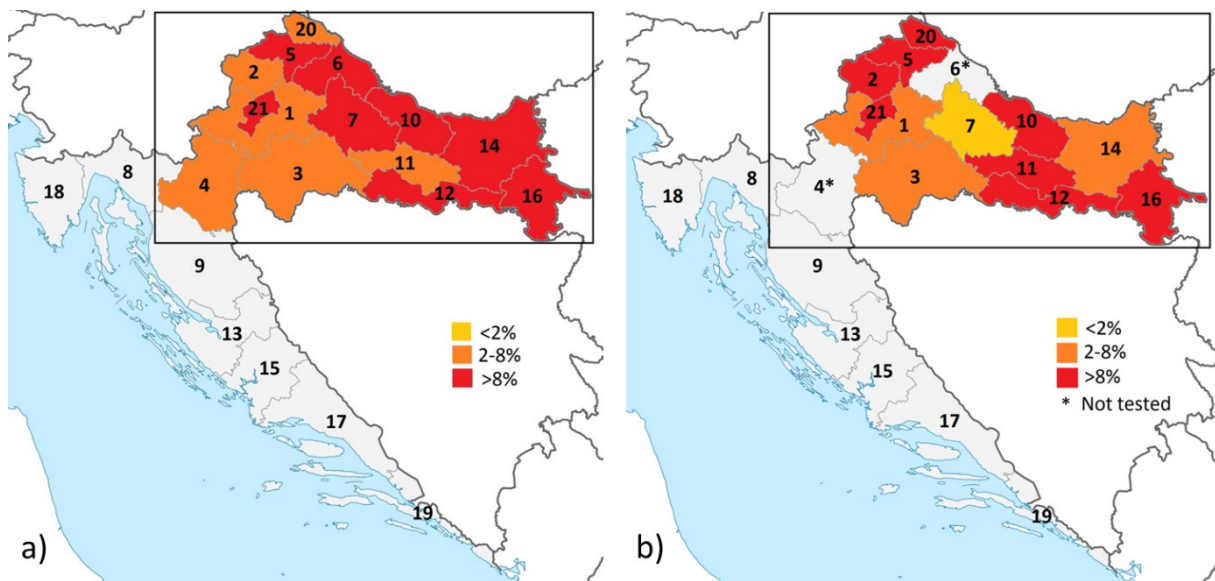
Figure 5. Seroprevalence of West Nile virus in horses (a) and poultry (b), Croatia, 2018
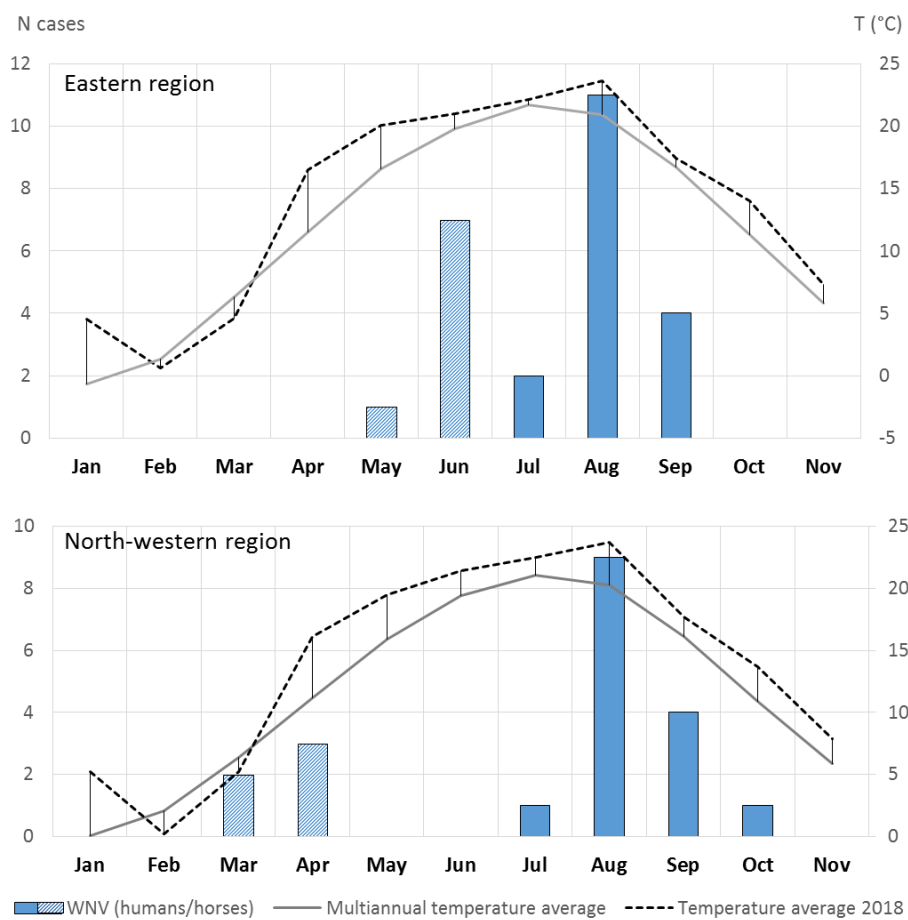

Figure 6. Relationship between temperature and WNV occurrence in Croatia, 2018