

**12. HRVATSKI KONGRES KLINIČKE MIKROBIOLOGIJE
9. HRVATSKI KONGRES O INFEKTIVNIM BOLESTIMA
s međunarodnim sudjelovanjem**

**12th CROATIAN CONGRESS OF CLINICAL MICROBIOLOGY
9th CROATIAN CONGRESS ON INFECTIOUS DISEASES
with international participation**



**KNJIGA SAŽETAKA
ABSTRACT BOOK**

ORGANIZATORI / ORGANIZERS

Hrvatski liječnički zbor
Croatian Medical Association

Hrvatsko društvo za kliničku mikrobiologiju
Croatian Society of Clinical Microbiology

Hrvatsko društvo za infektivne bolesti
Croatian Society for Infectious Diseases

Split, Hrvatska / *Croatia*
24. – 27. listopada 2019. / *October 24–27, 2019*

Molecular epidemiology of West Nile virus infections in Croatia

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West Nile virus (WNV) belongs to the family Flaviviridae, genus Flavivirus. Birds are natural reservoir for WNV, mosquitoes are vectors, while humans and other vertebrates are considered as accidental hosts. Infections caused by WNV in humans vary in the presentation, from asymptomatic infections to a severe neuroinvasive disease. In Croatia, WNV infections are detected in horses, birds and humans using serological and molecular methods. Currently, there are nine genetic lineages proposed for classification of WNV and the lineages 1 and 2 are responsible for extensive epidemics in animals and humans. The aim of this study was to present the molecular epidemiology of WNV infections in Croatia in the transmission seasons 2017 and 2018. Viral RNA was sequenced using Sanger method in four human samples in 2017 as well as eight human and one sample from a dead goshawk (*Accipiter gentilis*) in 2018. Analysis of the NS5 gene segment has shown that all the strains belong to the lineage 2 and show high similarity with strains detected in different hosts in other European countries. Grouping of nucleotide sequences from our country into different clusters regardless the transmission season, location and host was observed. This indicates to probably multiple introduction of the virus into Croatia, intensive circulation and local overwintering of the virus. For the viral RNA from the dead goshawk the whole genome was sequenced in 12 partially overlapping segments using Sanger method. The whole genome shows the highest similarity (99.8%) with strains detected the same year in birds from Germany. Despite the high similarity, a deletion of eight nucleotides was found in the 3' non-coding region of the genome which is not present in any of the available WNV sequences. Further studies at the molecular level of WNV detected in Croatia are needed to obtain better insight into the WNV molecular epidemiology and its local specificity

Usutu virus infections in Croatia: five years later

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Usutu virus (USUV) is a mosquito-borne arbovirus that belongs to the family Flaviviridae, genus Flavivirus. Birds are the natural reservoirs of the virus and mosquitoes of the genus *Culex* are the main vectors. Although human clinical cases were rarely reported, recent data from Italy showed that USUV infections may be more frequent than West Nile virus infections in some regions where these viruses co-circulate. The aim of this study was to present the occurrence, clinical symptoms and molecular epidemiology of USUV infections detected in Croatia. USUV was confirmed by detection of specific antibodies using ELISA and virus neutralization test (VNT) and/or detection of USUV RNA using RT-PCR. USUV RNA positive samples were further tested using Sanger sequencing. In Croatia, the first USUV seropositive human was detected in 2012. So far, six cases of human USUV neuroinvasive disease were confirmed. Three cases were detected in 2013 and three cases in 2018 transmission season, five years after the first ones. In five patients, infection was confirmed by detection of USUV neutralizing antibodies, while in one fatal case, USUV RNA was detected in a urine sample. Patients were from Zagreb/Zagreb County, Požega-Slavonia and Osijek-Baranja County. Two USUV seropositive horses were recorded 2011 in Zagreb and Sisak-Moslavina County. During the 2018 outbreak, USUV was detected for the first time in one dead blackbird (*Turdus merula*) from Zagreb County. USUV positive mosquito pools were recorded in the season 2016 in Zagreb (*Aedes albopictus*), 2017 in Međimurje County (*Culex pipiens*) and 2018 again in Zagreb (*Cx. pipiens*). Sequence was obtained from pooled sample collected in 2018. Phylogenetic analysis showed that Croatian strains detected in human, blackbird and *Cx. pipiens* pool belong to USUV Europe 2 lineage. Our results confirm the presence of USUV in Croatia. Further studies are needed to analyze the clinical significance and distribution of this emerging arboviral infection.

Neuroinvasive infection caused by Toscana virus – a neglected disease

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Toscana virus (TOSV) is an arthropod-borne virus, transmitted to humans by sandflies. Although majority of human TOSV infections are asymptomatic or presented as a non-specific febrile disease, neuroinvasive disease may also occur. TOSV is endemic in the Mediterranean countries with the highest infection rates during summer months. In Croatia, there are very few data on clinical cases of TOSV infection. We present a case report of TOSV infection detected in Croatian patient presented with aseptic meningitis.

In the summer 2018, a 21-year-old male patient from Middle Dalmatia was admitted to the Infectious Disease Department with a two-day history of fever (up to 38°C), headache, nausea, vomiting and fatigue. At admission, the patient was subfebrile (T=37.6°C) with normal vital signs.

Routine blood laboratory parameters were within normal range. Cerebrospinal fluid (CSF) analysis revealed a white blood cell count of 175 cells/mm³ with 76% lymphocytes, a protein level of 0.447 g/L (reference range 0.17-0.37) and a glucose level of 3.78 mmol/L (reference range 2.5-3.3). Brain computed tomography was normal.

Since the CSF findings were suggestive of aseptic meningitis, arboviral etiology was suspected. CSF and serum samples were tested for the presence of neuroinvasive arboviruses: tick-borne encephalitis, West Nile, Usutu, TOSV, Tahyna and Bhanja virus. High titers of both IgM (1000) and IgG (32000) to TOSV in a serum sample indicated acute TOSV infection. TOSV RNA was not detected in the CSF or serum sample. In addition, antibodies to other phleboviruses (sandfly fever Sicilian, Naples and Cyprus virus) were negative. The patient was treated with symptomatic therapy and recovered fully within five days.

The presented case highlights the need of increasing awareness of TOSV infection as a possible cause of aseptic meningitis/meningoencephalitis during the arbovirus transmission season.

Dual infection with West Nile virus and echovirus 9 in a patient with aseptic meningitis

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The seasonality, clinical and laboratory features of West Nile virus (WNV) neuroinvasive infection overlap with those of enteroviruses. We report a case of dual infection with WNV and echovirus 9 in a patient with aseptic meningitis. In August 2018, a 59-year-old woman with a history of arterial hypertension was hospitalized at the Clinic for Infectious Diseases on the third day of the illness. The disease was manifested by a daily fever up to 38.2°C, diffuse headaches and nausea without vomiting. On admission, her condition appeared severe. She was conscious, but she suffered from weakness and hypotonia. Neurologic examination revealed terminal neck stiffness. Routine laboratory parameters were normal. Cerebrospinal fluid (CSF) analysis showed pleocytosis (19×10⁶ cells/L) with 53% mononuclear cells and elevated protein level (0.412 g/L). Electroencephalogram showed slow diffuse dysrhythmia. PCR of CSF for HSV 1/2, enteroviruses, CMV, EBV, *B. burgdorferi* and *L. monocytogenes* were negative. High titer of both WNV IgM (ratio 3.36; >1.1 positive) and IgG antibodies (142.81 RU/ml; positive >22) of low avidity (avidity index 21%; low <40%) in serum sample as well as equivocal IgG antibodies (18.86 RU/ml) in CSF sample indicated WNV infection. RT-PCR of CSF and urine was negative for WNV RNA. Echovirus 9 was isolated from a stool sample. IgG antibodies to echoviruses were found in a serum sample, while CSF was negative. The patient was initially treated with acyclovir, ampicillin and ceftriaxone intravenously (until the completion of virology results) with a supportive (antiedematous) therapy. Third day into hospitalization, the patient was afebrile and without headache. The patient was monitored during three months and recovered fully. The presented case of acute WNV meningitis occurring concurrent with echovirus 9 infection suggest possible co-infections. Due to the overlapping seasonal activity, differential diagnosis of neuroinvasive infections should include both WNV and enteroviruses.

Prevalence of tick-borne encephalitis and Lyme-borreliosis in endemic regions of continental Croatia (2017-2019)

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Infections caused by tick-borne encephalitis virus (TBEV) and *Borrelia burgdorferi* are widely distributed in Europe. Coinfections with TBEV and borrelia are also reported. In Croatia, TBEV is endemic in north-western regions, however infections were reported in all continental counties. Borreliosis has the similar geographic distribution.

The aim of this study was to analyze the prevalence of TBE and Lyme-borreliosis in the Croatian mainland during the three transmission seasons.

From April 2017 to August 2019, 362 patients from continental Croatian regions presented with neuroinvasive disease (meningitis, encephalitis) were tested for TBEV and *B. burgdorferi*. Serological tests of serum and cerebrospinal fluid samples were performed using enzyme-linked immunosorbent assays (TBEV; Euroimmun, Lübeck, Germany; *B. burgdorferi*; Virotech, Rüsselsheim, Germany). Samples with positive TBEV IgM and IgG antibodies were further tested for IgG avidity (Euroimmun, Lübeck, Germany) to confirm acute/recent infection. *B. burgdorferi* antibodies were confirmed using an immunoblot test (Euroimmun, Lübeck, Germany).

Acute TBEV infection was confirmed by detection of IgM and low avidity IgG antibodies in 33 patients. Seven patients showed IgG antibodies of high avidity

indicating previous TBEV infection. Neuroborreliosis was confirmed in four patients, while 13 patients showed IgG antibodies to *B. burgdorferi*. In three patients, TBEV and *B. burgdorferi* co-infection was detected. Cases occurred from April to November, with majority reported in May/June/July (25/62.5%). Infections were detected in 12/21 Croatian counties. The highest prevalence was recorded in Međimurje (5/12.5%) and Koprivnica-Križevci County (4/10.0%). In Gorski Kotar, a small outbreak with six cases of TBEV infection was reported in 2019. Presented results indicate that TBE and borreliosis are still endemic continental Croatian counties. Sporadic infections and small outbreaks are continuously detected with seasonal distribution of cases.