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USUTU VIRUS: AN EMERGING FLAVIVIRUS IN EUROPE

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Abstract

Among various arthropod-borne viruses (arboviruses), the flaviviruses stand out with regard to their number, geographic distribution and importance in both human and veterinary medicine. West Nile virus is flavivirus, present endemic in many European countries as well as in Serbia where it circulates in horses, birds, humans and mosquitoes. Usutu virus (USUV) is flavivirus morphologically, antigenically, genetically and ecologically very similar to WNV, which circulates in neighbouring countries (Hungary, Croatia, Austria). The USUV is maintained in transmissible cycle between birds and mosquitoes mainly from the genus *Culex*. Mammals (humans, horses, rodents) can also be infected. Humans and other mammals are "dead end" hosts. Virus is isolated from numerous bird species. The USUV infections are asymptomatic in wild African birds, while for European birds, the virus is very virulent causing necrotizing focal encephalitis, degenerative myocarditis and fatal encephalitis. It is assumed that the virus was introduced into Europe by the migratory birds that have been infected by living or passing through endemic areas in Africa. First human cases were recorded in Italy in 2009. The genome of USUV was detected in cerebrospinal fluid of woman suffering from B-cell lymphoma with meningoencephalitis and in plasma of a female, who was subjected to a liver transplantation and subsequently developed fever, headache, and fulminant hepatitis which progressed to coma. In Austria, USUV infections were confirmed

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in people with a skin rash of unknown aetiology using plaque reduction neutralization test. The circulation of USUV has been proven in humans in many European countries by serological studies (Germany, Italy, and Croatia). Serological study performed in 2015 revealed that USUV is present in inhabitants of South Bačka District of Vojvodina, Serbia. Serum samples were tested using commercial ELISA IgG test for USUV and IgG antibodies against USUV were detected in 5% (4/88) of patients. The molecular investigation included 216 pools of mosquitoes collected in the period from June to September, in the South Bačka District. The USUV genome was detected in two mosquito pools (2/216). In human samples tested by RT PCR, USUV genome was not found.

Key words: USUTU virus, morphology, biology, diagnosis

USUTU VIRUS: NOVI FLAVIVIRUS U EVROPI

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Kratki sadržaj

Među Arbovirusima po brojnosti, rasprostranjenosti i značaju za humanu i veterinarsku medicinu izdvajaju se flavivirusi, RNK virusi koje prenose komarci i krpelji. Virus Zapadnog Nila (VZN) je flavivirus, endemski prisutan u mnogim evropskim zemljama, pa i u Srbiji gde cirkuliše u konjima, pticama, ljudima i komarcima. Usutu virus (USUV) je flavivirus morfološki, antigenski, genetski i biološki vrlo blizak VZN i cirkuliše u zemljama u okruženju (Austrija, Mađarska, Hrvatska). Virus se održava u transmisivnom ciklusu između ptica i komaraca uglavnom iz roda *Culex*. Sisari (čovek, konji, glodari) takođe mogu biti inficirani i slučajni su domaćini. Virus je izolovan iz različitih vrsta ptica. Kod afričkih ptica

infekcija je asimptomatska. Za evropske ptice virus je veoma virulentan i izaziva nekrotizirajući fokalni encephalitis, degenerativni miokarditis i fatalni encefalitis. Pretpostavlja se da su virus u Evropu donele migratorne ptice koje su se inficirale tokom boravka ili prolaska kroz endemska područja u Africi. U Italiji 2009. godine registrovan je prvi slučaj infekcije USUV kod ljudi. Genom USUV dokazan je u likvoru žene sa limfomom B ćelija, kao i iz plazme žene kojoj je transplantirana jetra i u koje su se razvili groznica, glavobolja, fulminantni hepatitis i koma. U Austriji kod osoba sa osipom na koži nerazjašnjene etiologije utvrđena je infekcija ovi virusom, neutralizacionim testom redukcije plakova. Serološke studije sprovedene na stanovništvu nekoliko evropskih zemalja ukazuju na prisustvo ovoga virusa kod ljudi (Nemačka, Italija, Hrvatska). Primenom metode ELISA uz korišćenje komercijalnog ELISA IgG testa na USUV, utvrđeno je prisustvo specifičnih IgG antitela protiv navedenog virusa kod 5% ispitanih uzoraka krvnog seruma ljudi (4/88). U periodu od juna do septembra 2015.godine ispitano je ukupno 216 zbirnih uzoraka komaraca prikupljenih na teritoriji Južnobačkog okruga. Sekvenca RNK USUV je dokazana u dva pula (2/216) komaraca. Testiranjem humanih uzoraka na USUV nisu dobijeni pozitivni rezultati.

Ključne reči: usutu virus, morfologija, biologija, dijagnostika

INTRODUCTION

Among various arthropod-borne viruses (arboviruses), the flaviviruses stand out with regard to their number, geographic distribution and importance in both human and veterinary medicine. They belong to the family *Flaviviridae*, genus *Flavivirus*. Flaviviruses are enveloped viruses with a positive sense single-stranded RNA genome, transmitted by mosquitoes and ticks. According to their antigenic characteristics, flaviviruses were classified into 8 serogroups: tick - borne encephalitis, Rio Bravo, Japanese encephalitis, Tyuleniy, Ntaya, Uganda S, dengue and Modoc (Calisher et al., 1989).

The Japanese encephalitis serogroup includes West Nile virus (WNV) which has now spread globally throughout Africa, Asia, Europe and America, Japanese encephalitis virus that is endemic in Southeast and East Asia, and Oceania, Marray Valley encephalitis virus prevalent in Australia, Saint Louis encephalitis virus spread over American continent, and Usutu virus (USUV). In recent years, a great expansion of WNV was confirmed. In just few years,

the virus has become endemic in the United States, involving thousands of human cases and hundreds of neuroinvasive disease cases reported annually. Endemic circulation of WNV has also been reported in many European countries. The results of serological and molecular studies revealed that WNV is present in Vojvodina and other regions of Serbia and that the virus circulates in horses, birds, humans and mosquitoes (Lupulović et al., 2011; Petrić et al., 2012; Petrović et al., 2013). No confirmed human cases of WNV infection were recorded in our country until 2012, when the first outbreak of WNV occurred in Serbia. Between August and October 2012, 58 people with WNV infection were hospitalized at the Institute of Tropical Medicine in Belgrade. Most of them (52) had neuroinvasive disease and 9 patients died (Popović et al., 2013). During the 2012 and 2013, 32 patients with WNV infection were treated at the Clinic for Infectious Diseases of the Clinical Centre of Vojvodina, and 17 of those had developed a neuroinvasive form of disease (Sević et al., 2015). Previously, WNV has been regarded as exotic virus from distant tropical countries with minor health importance. However, over the last several years, WNV has shown an increasing ability to spread beyond its established geographic ranges and has become an important public health concern in our country.

Increase in knowledge and understanding of WNV as an emerging human pathogen in our region extended our investigations to USUV, a virus taxonomically, morphologically, antigenically, genetically and ecologically very similar to WNV. USUV was first identified in 1959 in South Africa, when Mcintosh isolated the virus from *Culex neaevii* mosquitoes by intracerebral inoculation of new-born mice. The virus was named after the river Usutu in Swaziland. For decades after the discovery, the USUV was restricted to the African region, and it was considered to be unimportant in terms of pathogenicity. Increased attention was paid to USUV in 2001, when the first recognized outbreak of USUV outside of Africa occurred among blackbirds *Turdus merula* in Vienna, Austria (Weissenböck et al., 2002).

MORPHOLOGY OF USUV

Like other members of the family *Flaviviridae*, USUV is small, spherical virus with icosahedral symmetry. USUV virions are composed of a lipid envelope surrounding a nucleocapsid which harbours a positive single-stranded RNA genome with a length between 10,488 and 10,976 nucleotides. The untranslated, non-coding regions at 5' (5'UTR) and 3' (3'UTR) terminal ends of the genome flank the coding sequence. A single open reading frame encodes a polyprotein precursor that is co- and post-translationally processed into three

structural proteins named C (capsid protein), prM (precursor of the membrane protein M) and E (envelope protein) and 7 non-structural proteins with regulatory and enzymatic functions (NS1, NS2A, NS2B, NS3, NS4A, NS5B and NS5). Like in other flaviviruses, the adsorption of the virus to the cell is performed with E protein expressed on the surface of viral particles. During viral replication, the precursor polyprotein is cleaved by cellular and viral protease to the individual proteins (ICTV, 2016).

ISOLATION OF USUV

In the laboratory, the USUV multiplies in newborn mice up to 7 days of age, in which it causes fatal neurological disease. After inoculation, geese and chickens may have asymptomatic infection and occasionally excrete virus. The virus can be propagated in VERO E6, PK15, EGF, Hela cell cultures (Bakonyi et al., 2005). In infected cells, inclusions can be observed.

BIOLOGY OF USUV

The USUV is maintained in transmissible cycle between birds and mosquitoes mainly from the genus *Culex*. Birds are the reservoir (amplification host) and mosquitoes serve as vectors. The virus has been isolated from various species of mosquitoes: *Culex neavei*, *Culex perfuscus*, *Mansonia africana*, *Mansonia aurites* and *Aedes minutus* in Africa and *Aedes albopictus* and *Culex pipiens* in Europe. The infected mosquito transmits the infection to humans, but other mammals (horses, rodents) can also be infected. Humans and mammals are "dead end" hosts for USUV without significance for maintaining the virus in nature, as the low level of viraemia is usually not sufficient to allow the transmission of the virus to mosquitoes.

The USUV has been isolated from different African birds including *Bycanisters harpei*, *Andropadus virens*, *Turdus libomyanus*, and *Andropadus virens*. It has also been detected in number of European bird species as well. For example, in Italy, USUV was isolated in *Turdus merula, Sturnus vulgaris, Garrulus glandarius, Pica pica* (Tamba et al., 2011) In Austria, it was detected in *Turdus merula*, *Turdus philomelos*, *Strix nebulosa*, *Parus caeruleus*, *Passer domestic*us, *Parus major*, *Sitta europea*, *Erithacus rubeculain (*Weissenböck et al., 2003). In Germany, the presence of USUV was confirmed in *Turdus merula, Sturnus vulgaris, Serinus canaria domestica, Passer domesticus, Strix nebulosa, Alcedo atthis* (Jöst et al., 2011). In Switzerland, the virus was found in *Passer domesticus, Turdus merula, Passer caeruleus, Carduelis chloris, Erithacus rubecula,*

Aegolius funereus, Strix nebulosa lapponica, *Surnia ulula, Glaucidium passerinum (*Steinmetzd et al., 2011). In Hungary, it was detected in *Turdus merula (*Bakonyi et al., 2007).

The USUV has been present in Africa for a substantial period of time. Long-term co-evolution of USUV and its avian hosts resulted in natural selection of birds able to survive and become resistant to infection. As a consequence, the USUV infections are asymptomatic in wild African birds, while for European birds, the virus is very virulent causing necrotizing focal encephalitis, degenerative myocarditis and fatal encephalitis (Bakonyi et al., 2007). It is assumed that the virus was introduced into Europe by migratory birds that have been infected by living or passing through endemic areas in Africa. The virus also infects rodents. It was isolated for the first time from rodent *Praomys sp.* in Central African Republic.

HUMAN CASES OF USUV INFECTION

The first human case of infection with USUV has been registered in the Central African Republic, where the virus was isolated from serum sample of patient with fever and skin rash. Another case has been registered in Burkina Faso in the 10-year-old patient with fever and jaundice.

The first human case of USUV infection in Europe was recorded in 2009 in Italy. The virus was detected in cerebrospinal fluid (CSF) of a 60-year-old woman suffering from B-cell lymphoma, with meningoencephalitis, using RT-PCR assay with primers specific for NS5 and prM genes. Sequencing of amplification products revealed 98% homology with strains Vienna-2001 and Budapest-2005 (Pekorari at al., 2009). In the same year, the second case of human USUV infection was registered in Italy. It was a female, age 40, who was subjected to a liver transplantation and subsequently developed fever, headache, and fulminant hepatitis which progressed to coma. USUV was isolated from plasma sample on VeroE6 cell culture and identified by real-time RT PCR test. The virus genome was completely sequenced and the virus was named Bologna (Cavrini et al., 2009). It is interesting that both patients were from the Northeast region of Italy called Emilia-Romagna, where the first transmission of Chikungunya virus in Europe was established. Another three human cases of USUV infections were confirmed also in Italy, when the genome of the USUV was found in CSF samples of 3 out of 44 patients with meningoencephalitis (Cavrini et al., 2011). In Austria, USUV infections were confirmed by plaque reduction neutralization test in 25% of 203 people with an increased risk of USUV infection and a skin rash of unknown aetiology (Weissenböck et al., 2007).

SEROLOGICAL INVESTIGATIONS OF USUV IN HUMANS

Serological studies performed in several European countries indicated the circulation of USUV among humans. In a study conducted in southwest Germany, the antibodies against USUV were found in one out of 4,200 blood donors using enzyme linked immunosorbent assay (ELISA), immunofluorescent test (IFT) and neutralization test (Allering et al.,2012*)*. In Italy, USUV IgG antibodies were found in 4 out of 359 blood donors via ELISA test (Gaibani et al., 2012). In Croatia, antibodies against USUV were detected in 3 out of 95 patients with fever and neuroinvasive symptoms, using virus neutralization assay (Vilibic - Cavlek et al., 2014). Seroepidemiological investigations detected the virus in mosquitoes and birds in Austria, Germany, Italy, Spain, Switzerland, Hungary, Poland, Belgium, Greece, the Czech Republic and England. During the study of WNV in horses, antibodies against USUV were discovered by neutralization test in serum samples of horses also positive to anti-WNV antibodies (Lupulović et al., 2011*)*.

SEROLOGICAL INVESTIGATIONS OF USUV IN VOJVODINA

Serological investigation performed in 2015 revealed that the USUV is present in inhabitants of South Bačka District of Vojvodina, Serbia. The study included 88 persons with risk factors for infection with arboviruses transmitted by mosquitoes. Serum samples were tested using commercial ELISA IgG test for USUV ("Euroimmun", Germany), strictly according to the manufacturer's recommendations. IgG antibodies against USUV were detected in 5% (4/88) of patients (Hrnjaković Cvjetković et al., 2014).

MOLECULAR INVESTIGATION OF USUV IN VOJVODINA

Molecular investigations for the presence of USUV specific RNA in pooled samples of mosquitoes and human samples were conducted during the 2015. The investigation included 216 pooled samples of mosquitoes collected in the period from June to September in the South Bačka District. Pooled samples of mosquitoes were homogenized and viral RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Germany) kits. For the detection of USUV genome, real-time RT PCR was applied. Amplification was performed with oligonucleotide primers and TaqMan probe ("Invitrogen", USA) specific for the NS5 gene, using *SuperScript* III *One-Step RT-PCR* ("Invitrogen", USA) on Applied Biosystems 7500 thermocycler ("Applied Biosystems", USA). The USUV

genome was detected in two mosquito pools (2/216). Samples of patients with neurological symptoms and symptoms of fever (39 serum samples and 20 samples of CSF) were also examined by real-time RT PCR test. None of the samples tested were positive for USUV RNA (Hrnjaković Cvjetković et al., 2015).

DIAGNOSIS OF USUV INFECTION

Serological diagnosis of USUV infection is often difficult due to extensive cross-reactivity between different flaviviruses, particularly in geographic regions where circulation with other flaviviruses, such as WNV and tick-borne encephalitis virus (TBEV) occur. The cross-reactivity is higher for IgG than for IgM detection (Makino et al., 1994). According to available information, there are only commercially available USUV-specific IgG ELISA tests, at the moment. Consequently, development of commercially available USUV-specific IgM ELISA test is needed most urgently. The neutralization test can be used as specific and sensitive tool to overcome the flavivirus cross-reactivity. However, the test is time-consuming and labour-intensive. It is performed using cell cultures and live viruses and requires the biosafety level III facilities, which are available in limited number of laboratories. An additional problem in serological diagnosis of arboviral infections is a long-term persistence of IgM antibodies in serum, sometimes months after the infection (Solomon, 2004).

Molecular tests are now accepted as standard tests for diagnosis of USUV infections in acute phase of the disease. At present, the real-time RT PCR tests are used most frequently. Molecular tests allow the most specific, sensitive and rapid detection of USUV genome in serum and CSF samples within the first days after the onset of infection.

CONCLUSION

The results of many studies indicated that USUV circulates in mosquitoes, birds and humans in Europe. USUV is active in Vojvodina. Genome of USUV was detected in two pools of mosquitoes collected in South Bačka District. Serological investigation showed that USUV is active among humans. The assessment of actual risks associated with USUV for humans and animals in Vojvodina strongly requires further investigations. Large-scale veterinary, human and entomology based surveillance programs should be established to prevent the emergence of USUV in Vojvodina.

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