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CASE REPORT

First cases of human Usutu virus neuroinvasive infection in Croatia, August–September 2013: clinical and laboratory features

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Abstract Few reports of human Usutu virus (USUV) infection have been reported to date. We describe the first three patients with USUV neuroinvasive infection in Zagreb and its surroundings from 30 August to 7 September 2013 during a West Nile virus (WNV) outbreak. Patients were aged 29, 56, and 61 years. The two older patients had several comorbidities (arterial hypertension, hyperlipidemia, and diabetes mellitus). All patients presented with meningitis and meningoencephalitis closely resembling WNV neuroinvasive disease. The main clinical features in all patients were headache, fever, nuchal rigidity, hand tremor, and hyperreflexia. Neuroimaging studies were normal and electroencephalography (EEG) revealed diffusely slow activity. The 29 years old, a previously healthy female patient, was deeply somnolent and disoriented for 4 days. Her recovery was slow and even 10 weeks after disease onset, she had memory and speech-fluency difficulties. The other two patients recovered promptly. USUV IgG antibodies were detected in all patients by ELISA with seroconversion documented in two of them. Titers of USUV-neutralizing antibodies were 10, 80, and 10, respectively. Because USUV and WNV share many clinical characteristics, USUV infection could be misdiagnosed as WNV. Testing for USUV should be considered in all suspected cases of meningoencephalitis, especially in areas where both viruses cocirculate.

Keywords Usutu virus · Neuroinvasive disease · Humans · Croatia

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Introduction

Usutu virus (USUV) is an arthropod-borne virus of the family *Flaviviridae*, genus *Flavivirus*, Japanese encephalitis serocomplex. The virus emerged in Europe in 1996 and has caused considerable avian mortality (Weissenböck et al. 2002, 2013; Bakonyi et al. 2007; Steinmetz et al. 2011), especially in blackbirds (Chvala et al. 2004).

The pathogenicity of USUV in humans is not fully understood, because few clinical cases of human infection have been reported to date. Two cases of USUV fever with rash and jaundice were reported in the Central African Republic and Burkina Faso in 1981 and 2004 (Nikolay et al. 2011). USUV-neutralizing antibodies were detected in 25 % of Austrian patients with rash exposed to mosquitoes in endemic regions during 2006 (Weissenböck et al. 2007) and in 2009, USUV neuroinvasive disease was confirmed in two immunocompromised Italian patients (Pecorari et al. 2009; Cavrini

et al. 2009). Serologic evidence of USUV infection (IgG antibodies) in four Italian blood donors (2011), as well as asymptomatic acute infection in a German blood donor (2012), indicates that the virus circulates among humans in Europe (Gaibani et al. 2012; Allering et al. 2012).

We report the first clinical cases of human USUV neuroinvasive infection in Croatia detected during a West Nile virus outbreak in 2013.

Case reports

Patients' demographic, epidemiological, clinical, and laboratory data are presented in Tables 1, 2, and 3. None of the patients reported a history of vaccination against tick-borne encephalitis, yellow fever, or Japanese encephalitis.

Case 1

On 2 September 2013, a previously healthy 29-year-old female from Zagreb was hospitalized because of a pulsating parietooccipital headache lasting for 4 days and fever up to 39 °C for 2 days. Before hospitalization, the patient was not receiving any medications. She had not left Zagreb for 4 weeks before the onset of illness, and she denied any tick bites.

At admission, she was acutely ill-appearing with normal vital signs, lethargic but arousable, and fully oriented. She had nuchal rigidity, pronounced intention hand tremor, moderate dysmetria, hyperreflexia (non-sustained clonus, 4^{+}) at all levels, and bilateral extensor plantar response. Examination of the cranial nerves, muscle tone, power, and sensorium was normal. Physical examination was unremarkable. Intravenous acyclovir was started. For the following 3 days, she was febrile, lethargic, and disoriented with severe headache, nausea, vomiting, intention hand tremor, and dysmetria. Non-contrast brain CT and MRI were normal. Cerebrospinal fluid (CSF) samples were negative for herpes simplex virus (HSV) 1/2 and enteroviruses by PCR. Serology for syphilis, HIV, and *Borrelia burgdorferi* were negative. Acyclovir was discontinued. From the twelfth day of disease, her condition started to improve. The patient was discharged from the hospital with normal neurological findings. She was amnestic for the first 7 days of hospitalization.

However, her recovery was slow and even 10 weeks after disease onset, she still had headaches. Psychological tests performed after 10 weeks showed difficulties in declarative memory (normal immediate recall but impaired

Table 1 Main demographic, epidemiological, and clinical data of three patients with Usutu virus neuroinvasive disease in Croatia

Characteristic	Case 1	Case 2	Case 3
Demographic data			
Age	29	61	56
Gender	Female	Male	Male
Epidemiological data			
Place of residence	Urban (Zagreb)	Rural (Hrvatsko Zagorje)	Urban (Velika Gorica)
Traveling (4 weeks before)	No	No	No
TBE/YF vaccination	No	No	No
Clinical presentation			
Date of disease onset	August 30, 2013	September 7, 2013	September 4, 2013
Days to hospitalization	4	3	7
Main signs and symptoms	Headache, fever, somnolence, disorientation, nausea, vomiting, nuchal rigidity, intention hand tremor, hyperreflexia at all levels, extensor plantar response, dysmetria	Headache, transient diplopia, fever, somnolence, nuchal rigidity, tongue tremor, intention hand tremor, hyperreflexia at all levels, extensor plantar response	Headache, fever, nuchal rigidity
The highest body temperature	39.0 °C	39.0 °C	38.6 °C
The lowest GCS	14	15	15
Comorbidities	None	Arterial hypertension, hypertensive cardiomyopathy, permanent atrial fibrillation, diabetes	Arterial hypertension, coronary heart disease, diabetes
Length of stay (days)	9	13	6
Outcome at discharge (GOS)	4	5	5

TBE tick-borne encephalitis, *YF* yellow fever, *GCS* glasgow coma score, *GOS* glasgow outcome score

Table 2 Laboratory and neuroimaging features of three patients with Usutu virus neuroinvasive disease in Croatia

Parameter	Case 1	Case 2	Case 3	Reference range
Cerebrospinal fluid (CSF) examination	Day 4	Day 4	Day 7	
Cell count/mm^3	240	167	80	0–5
Polymorphonuclear/mononuclear cells	4/96 %	45/55 %	20/80 %	Mononuclear 100 %
Proteins (g/l)	0.68	1.93	0.81	0.17–0.37
Glucose (% of blood glucose)	73	62	65	60–70
Bacteriology result	Negative	Negative	Negative	–
Serum examination	Day 4	Day 4	Day 7	
C-reactive protein; CRP (mg/L)	3.5	26.2	5.2	<5.0
White blood cells; WBC (×10^9/L)	7.8	11.7	10.4	4.0–10.0
Platelets (×10^9/L)	156	223	264	100–400
Red blood cells; RBC (×10^{12}/L)	3.90	4.76	5.37	4.4–5.8
Hemoglobin (g/L)	127	138	154	120–180
Bilirubin (μmol/L)	12.4	19.8	19.1	3.0–20.0
Aspartate-aminotransferase; AST (U/L)	14	19	76	11–38
Alanine-aminotransferase; ALT (U/L)	11	18	86	12–48
Gamma-glutamyltransferase; GGT (U/L)	29	57	255	11–55
Lactate dehydrogenase; LDH (U/L)	97	165	157	<241
Brain computed tomography; CT	Normal (day 4)	Normal (day 4)	Not done	–
Brain magnetic resonance imaging; MRI	Normal (day 12)	Not done	Not done	–
Electroencephalogram; EEG	Irregular, diffuse slowing (day 5)	Diffuse slowing (day 4)	Diffuse slowing (day 7)	–

recent verbal and visual memory) and decreased fluency of spontaneous speech.

Case 2

On 9 September 2013, a 61-year-old male was hospitalized with 3 days of headache, fever up to 39 °C, and neck pain. On the first day of the fever, he had vertical diplopia which disappeared spontaneously after several hours. The patient was treated at home with ibuprofen. Previous medical history disclosed arterial hypertension, permanent atrial fibrillation, and diabetes. The patient was living in a rural part of Hrvatsko Zagorje, did not report recent travel, and denied any tick bites.

Upon admission, he was febrile (38.1 °C), cardiocirculatory and respiratory stable, ambulatory, alert, and fully oriented. Neurological examination revealed a stiff neck, tongue tremor, intention hand tremor, hyperreflexia (4$^+$) at all levels, and extensor plantar response on the left side. Examination of the cranial nerves, muscle tone, power, and sensorium was normal. Physical examination revealed bilateral facial and conjunctival redness and irregular pulse from permanent atrial fibrillation. Non-contrast brain CT was normal. Lumbar puncture revealed pleocytosis with 55 % mononuclear cells, high protein levels, and normal glucose. Acyclovir, ampicillin, and ceftriaxone were started. For the next 3 days, the patient was febrile, somnolent, and lethargic with intention hand tremor. EEG registered diffuse slowing.

Table 3 Serology results of three patients with Usutu virus neuroinvasive disease in Croatia

Case	Day tested	USUV IgG (RU/ml)	USUV VNT (titre)	WNV IgM (ratio)/ IgG (RU/ml)	WNV VNT (titre)	DENV IgM (ratio)/ IgG (RU/ml)	TBEV IgM/ IgG (titre)	JEV IgM/ IgG (titre)	YFV IgM/ IgG (titre)
1	3	Neg (<2)	–	Pos (1.25)/neg (<2)	–	Equiv (1.06)/neg (12)	Neg/neg	Neg/neg	Neg/neg
	45	Pos (60)	10	Pos (2.39)/neg (10)	Neg	Equiv (0.95)/equiv. (20)	Neg/neg	Neg/neg	Neg/neg
2	5	Neg (5)	–	Pos (2.99)/neg (4)	–	Equiv (1.03)/neg (14)	Neg/neg	Neg/neg	Neg/neg
	12	Pos (28)	10	Pos (3.03)/equiv. (16)	5	Pos (1.27)/neg (10)	Neg/neg	10/neg	Neg/neg
3	9	Pos (108)	80	Pos (1.50)/pos (90)	20	Neg (0.27)/pos (68)	Neg/32	Neg/10	Neg/10

USUV Usutu virus, *DENV* dengue virus, *WNV* West Nile virus, *TBEV* tick-borne encephalitis virus, *JEV* Japanese encephalitis virus, *JEV* yellow fever virus, *ELISA* enzyme-linked immunosorbent assay, *VNT* virus neutralization assay, *IFA* indirect immunofluorescence assay, *RU/ml* relative units/ml

Antimicrobial medications were discontinued after obtaining negative HSV1/2 and *Listeria monocytogenes* PCR and negative bacteriology.

There was a gradual improvement and after 13 days, the patient was discharged in good condition without any neurological deficits. Eight weeks after hospitalization, he had no complaints.

Case 3

On 10 September 2013, a 56-year-old male was hospitalized with a headache and fever up to 38.6 °C lasting for 7 days. At home, he was taking acetaminophen, ibuprofen, and aspirin. Past medical history revealed arterial hypertension, coronary heart disease, hyperlipidemia, and diabetes. He was living in Velika Gorica (urban surroundings of Zagreb), did not report recent travel, and denied tick bites.

At admission, the patient was ambulatory, afebrile with normal vital signs, alert, and fully oriented with only nuchal rigidity in a neurological exam. Lumbar puncture revealed predominantly mononuclear pleocytosis with elevated protein and normal glucose level. EEG was diffusely slow. The patient received symptomatic therapy. For the next 3 days he was afebrile with a gradually decreasing headache. He recovered fully by the end of the 6-day hospitalization and 4 weeks after hospitalization, he reported no complaints.

Virology results

CSF of all three patients was tested for the presence of the flavivirus RNA by conventional reverse transcriptase (RT)-PCR that amplifies the 10,090–10,832-nt region (Weissenböck et al. 2002), as well as by real time RT-PCR (Johnson et al. 2010). All three samples were negative using both methods.

Serology results are presented in Table 3. At initial screening by ELISA (Euroimmun, Lübeck, Germany), WNV IgM antibodies were found in cases 1 and 2, along with IgG antibodies in case 3, indicating acute WNV infection. However, cases 1 and 2 were both WNV IgG negative in the initial and paired serum samples; therefore, they were further tested for USUV. USUV IgG antibodies were detected using ELISA (Euroimmun, Lübeck, Germany) in all patients (in cases 1 and 2, USUV IgG seroconversion was demonstrated). To exclude cross-reactive antibodies induced by other autochthonous or imported flaviviruses and/or vaccination, samples were additionally tested for dengue virus (DENV) using ELISA (Euroimmun, Lübeck, Germany), as well as tick-borne encephalitis virus (TBEV), Japanese encephalitis virus (JEV), and yellow fever virus (YFV) using an indirect immunofluorescence assay (IFA; Flavivirus mosaic, Euroimmun, Lübeck, Germany). In two samples (cases 1 and 2), equivocal/positive IgM response to DENV and equivocal IgG response to DENV and WNV was observed. In the third sample (case 3), IgG antibodies cross-reacted with all tested flaviviruses. USUV infection was confirmed by virus neutralization test (VNT) at the OIE Reference Centre for West Nile Disease, Istituto Zooprofilattico Sperimentale "G. Caporale", Teramo, Italy. USUV-neutralizing antibodies were confirmed in all patients. In two patients, USUV antibodies cross-reacted with WNV in VNT but showed higher titer against USUV than WNV, which indicates USUV infection.

Discussion

In Croatia, USUV-neutralizing antibodies were sporadically detected in horses (2011) (Barbic et al. 2013) and humans (2012) (Vilibic-Cavlek et al. 2014b) but no human clinical cases have been reported to date. Patients presented in this study emerged in Zagreb and its surroundings from 30 August to 7 September 2013, during an outbreak of WNV infection (Vilibic-Cavlek et al. 2014a). The clinical presentation was similar to WNV neuroinvasive disease, as well as USUV neuroinvasive disease in two cases reported from Italy (Pecorari et al. 2009; Cavrini et al. 2009). In contrast to the normal CSF clinical-chemical results found in the first Italian patient (Pecorari et al.), cases presented in this report had mostly mononuclear pleocytosis, elevated protein levels, and normal CSF glucose.

Similar to Italian reports (Cavrini et al. 2009; Pecorari et al. 2009), our cases indicate that age and comorbidities may have a role in the pathogenicity of USUV. Two of our patients were over 55 years of age and reported several comorbidities, but they recovered promptly. However, the 29-year-old patient was previously healthy and had a more severe form of disease with long-term headaches, memory, and speech difficulties similar to WNV neuroinvasive disease (Sejvar et al. 2008).

Because all three patients tested positive for WNV IgM antibodies at the initial screening, WNV neuroinvasive disease was suspected. However, there was no documented WNV IgG seroconversion. Within a vector-borne flaviviruses national surveillance program established in Croatia in 2011, reactive WNV samples are routinely tested for potential cross-reactivity to other flaviviruses, especially those reported in Croatia in humans or animals, such as USUV, TBEV, and DENV. Using ELISA, USUV IgG antibodies were detected in all samples (cases 1 and 2 seroconverted) and confirmed with a VNT.

Criteria for the diagnosis of USUV infections in humans are similar to those of WNV. According to ECDC and CDC definitions (ECDC 2009) of neuroinvasive WNV disease, serological evidence of infection in addition to clinical criteria confirms the diagnosis of neuroinvasive disease. Because of a high level

of cross-reactivity between flaviviruses, neutralization tests are the "gold standard" of flavivirus serology. Despite a high degree of specificity of VNTs, cross-neutralization within the same serocomplex is possible (Yeh et al. 2012; Stiasny et al. 2013; Beck et al. 2013). Because WNV and USUV are both members of the Japanese encephalitis serocomplex and have approximately 80 % of identical amino acids in the E protein (the major target of neutralizing antibodies), cross-reactivities are not only observed in ELISA but also in VNT. Cross-reactivity studies between USUV and WNV have indicated that the antibody titers to the homologous antigens were four to eight times higher than related viruses (Yeh et al. 2012; Stiasny et al. 2013; Beck et al. 2013). Viruses from different serocomplexes such as TBEV, DENV, and YFV only have about 40 % identical amino acids in the E protein; as a result, cross-reactivity is detectable in ELISA but usually not observed in VNT (Kuno et al. 2003; Stiasny et al. 2013). Serologic results of our patients were similar to these previous studies. A high level of cross-reactivity was observed with WNV and USUV. All samples cross-reacted in ELISA and two samples cross-reacted in VNT, with higher neutralizing antibody titers against USUV than against WNV (10 vs. 5 and 80 vs. 20), indicate USUV infection. In case 2, the second sample was taken only 1 week after the first sample, which could explain a lower USUV titer (twofold higher than WNV). In the third case, a fourfold higher titer of USUV-neutralizing antibodies compared with WNV confirmed USUV infection (Yeh et al. 2012; Stiasny et al. 2013; Beck et al. 2013).

In conclusion, our results indicate that USUV infection could be misdiagnosed as WNV because of similar clinical symptoms. It is important to raise clinicians' awareness of USUV. In areas where viruses cocirculate in humans or animals, all suspected neuroinvasive cases should be tested for WNV as well as for USUV. In addition, detection of USUV neuroinvasive disease in a young, previously healthy patient highlights that USUV should be considered even in patients without comorbidities.

Conflict of interest The authors declare that they have no conflict of interest.

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