



Epidemics of the central nervous system infections caused by West Nile virus in the territory of the South Bačka District, Vojvodina, Serbia

Epidemija infekcija centralnog nervnog sistema virusom Zapadnog Nila na teritoriji Južnobačkog okruga, Vojvodina, Srbija

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Abstract

Background/Aim. West Nile virus (WNV) is a neurotropic RNA virus particle which belongs to the *Flaviviridae* family, genus *Flavivirus*. It is sustained in arthropods within the transmission cycle between the mosquitoes and birds. Most commonly (80% of cases) WNV infections are asymptomatic among people. Less than 1% of patients develop neuroinvasive forms of the disease – meningitis, encephalitis, or acute flaccid paralysis. The aim of the research was to determine most common clinical and laboratory manifestations, to emphasize the presence of comorbidities and outcomes of treatment among patients with WNV infection. **Methods.** This retrospective study, which was conducted in the period from January 1, 2012 to December 31, 2013, evaluated 32 patients who were diagnosed with WNV infection based on clinical findings, laboratory, and serological tests. To assess statistical significance we used χ^2 , and *t*-test. **Results.** The study involved 22 (69%) males and 10 (31%) females aged from 31 to 65 years. On admission, there were 16 (50%) febrile

individuals, 27 (84.4%) with positive meningeal signs, 17 (53.2%) with pathological neurological signs, and 10 (31.3%) with consciousness disorders. WNV infection was confirmed by the method enzyme linked immuno sorbent assay (ELISA) in all the patients, while Reverse Transcription Polymerase Chain Reaction (RT-PCR) test was positive in 3 (30%) of the tested patients. Cardiovascular comorbidities dominated in 7 (21.9%) of the cases. Full recovery was accomplished in 87.5 % of the cases. **Conclusion.** The results of our study show that the absence of meningeal signs and fever on the day 7 of hospital treatment are indicators of good course and prognosis of neuroinvasive forms of WNV infection. Comorbidities do not increase the risk of disease. ELISA test is a sovereign diagnostic method. In most cases, after the administered symptomatic therapy, the complete recovery of patients was achieved.

Key words:

west nile virus; central nervous system viral diseases; diagnosis; treatment outcome; prognosis; serbia.

Apstrakt

Uvod/Cilj. Virus *West Nile* (WNV) je neurotropna RNA virusna partikula koja pripada porodici *Flaviviridae*, rodu *Flavivirus*. Održava se u artropodama u okviru transmisivnog ciklusa između komaraca i ptica. WNV infekcije su asimptomatske kod 80% ljudi. Manje od 1% inficiranih osoba razvije neuroin vazivni oblik bolesti, meningitis, encefalitis ili akutnu flakcidnu paralizu. Cilj rada bio je da se utvrde najčešće kliničko-laboratorijske manifestacije, prisustvo komorbiditeta i ishod lečenja kod obolelih od WNV infekcije. **Metode.** U ovu retrospektivnu studiju, sprovedenu u periodu od 1. 1. 2012. do 31. 12. 2013. godine, bila su

uključena 32 bolesnika, kojima je na osnovu kliničkog nalaza, laboratorijskih i seroloških analiza dijagnostikovana WNV infekcija. U svrhu ocene statističke značajnosti korišćeni su χ^2 i *t*-test. **Rezultati.** U studiju je bilo uključeno 22 (69%) bolesnika muškog pola i 10 (31%) žena, starosti 31 do 65 godina. Povišenu temperaturu na prijemu imalo je 16 (50%) bolesnika, pozitivne meningealne znake 27 (84.4%), patološke neurološke znake 17 (53,2%), a poremećaj stanja svesti 10 (31,3%). WNV infekcija je kod svih obolelih potvrđena metodom *enzyme linked immuno sorbent assay* (ELISA). Istovremeno *reverse transcription polymerase chain reaction* (RT-PCR) test bio je pozitivan kod 3 (30%) testirana bolesnika. Od komorbiditeta, najčešće su registrovane kar-

diovaskularne bolesti, kod 7 (21,9%) bolesnika. Potpuni oporavak postignut je kod 87,5% bolesnika. **Zaključak.** Rezultati naše studije pokazali su da su odsustvo febrilnosti i pozitivnih meningealnih znakova sedmog dana hospitalizacije pokazatelji dobrog ishoda neuroinvasivnih oblika WNV infekcije, a da prisustvo komorbiditeta ne povećava rizik od obolevanja od ove bolesti. ELISA predstavlja vr-

hunsku metodu za postavljanje dijagnoze. Kod najvećeg broja obolelih simptomatska terapija dovodi do potpunog oporavka.

Ključne reči:
virus zapadnog nila; nervni sistem, centralni, virusne bolesti; dijagnoza; lečenje, ishod; prognoza; srbija.

Introduction

West Nile Virus (WNV) is a small neurotropic RNA virus particle that belongs to the *Flaviviridae* family, genus *Flavivirus*. It is sustained in arthropods within the transmission cycle between the mosquitoes and birds. WNV is the most widely spread arbovirus type in the world¹.

Birds are natural reservoirs and hosts of WNV infection, while human population and other mammals could be occasionally infected after being bitten by infected mosquitoes². WNV was originally identified in 1937 in the endemic region of Uganda in a female patient with moderate febrile condition. It is the cause of sporadic infection cases in Europe and epidemics in endemic regions of Africa, southern Asia, and northern Australia. Starting from 1996, the WNV infection has been getting in significance in western countries causing massive epidemics or smaller cluster epidemics of WNV encephalitis in Europe²⁻⁴.

In the United States of America (USA), the WNV was recognised for the first time in 1999 when it became the key public health problem spreading across several countries. Today, this flavivirus represents the leading cause of neuroinvasive arboviral disease in the USA and it is responsible for the occurrence of focal seasonal epidemics. Until 2010 almost 1.8 million people in the USA were infected with WNV, and 12,851 cases of meningoencephalitis and 1,308 lethal outcomes of infection were reported^{2,4,5}.

In 80% of cases, the WNV infection is asymptomatic. The symptoms occur in insignificant number of patients (around 20%), most frequently in the form of acute system febrile condition – West Nile fever. It is believed that in clinical manifested cases the incubation period lasts from 3 to 14 days. Monitoring of the described epidemics indicates the presence of febrile condition (body temperature above 39°C accompanied by fatigue, anorexia, nausea, vomiting, myalgias, arthralgia, headache, maculopapulose rash and swollen lymph glands). The disease lasted for 3 to 6 days. In the epidemics occurring before 1999, there were also reported myocarditis, pancreatitis, and fulminant hepatitis cases. Less than 1% of patients with WNV infection develop a neuroinvasive form of disease that is manifested as meningitis, encephalitis, or acute flaccid paralysis and accompanied by higher morbidity and mortality⁴⁻⁶.

Transitional viremia occurs in patients infected with WNV directly prior to the occurrence of initial symptoms, *ie* in the early disease stage (5–14 days after inoculation) and it lasts for 4 to 9 days. The production of neutralising antibodies leads to termination of viremia and transiting of the disease in the symptomatic phase. Prolonged viremia is found in immunocompromised patients who cannot develop an ade-

quate immune response in the presence of virus particles. Specific immunoglobulins of IgM class are detected in the patient's serum 2 to 8 days after the occurrence of initial disease symptoms and this is accompanied with the increase of WNV-specific IgG antibodies⁵.

WNV genome is a single-stranded positive-polarity ribonucleic acid (RNA) molecule with approximately 11,000 nucleotides built up by 3 structural and 7 non-structural proteins. Out of five WNV lineages, lineages 1 and 2 are most widely spread in the world. Until 2004, only the WNV of lineage 1 circulated across Europe. That same year the WNV particle of lineage 2 was isolated for the first time in Hungary in the sample taken from the infected goshawk⁷.

The establishing of etiological WNV infection diagnosis is based on a routine use of serological tests: enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay (IFA), neutralization test (NT), and hemagglutination-inhibition test. The neutralization tests is considered most specific, it is difficult to perform, time consuming and it can be performed only in Biosecurity Level 3 (BSL-3) laboratories. Serological ELISA test is currently most widely used since it is fast, easy to perform, cheap and it enables detecting of immunoglobulin, *ie* IgM and IgG WNV antibodies⁸. The reverse transcription polymerase chain reaction (RT-PCR) is used to detect viral RNA during the acute disease phase. Sequencing of RT-PCR products can confirm the presence of WNV particle⁹.

WNV is transferred onto people primarily *via* infected mosquito's bites (genus *Culex*)^{5,9,10}.

The WNV infection epidemics in human population in the territory of the Republic of Serbia were reported for the first time in the period from August to October 2012 in northern parts of the country, including the AP Vojvodina^{3,7,11}.

The detection and understanding of a complex group of factors that condition the creating of the focus of WNV infection and consequential spreading of this pathogen in the environmental are of the key significance for forecasting and mitigating of future epidemics¹.

The aims of this retrospective study were to determine the most frequent subjective health problems and clinical manifestations in patients with neuroinvasive forms of WNV infection, the presence of comorbidities in patients, the significance of laboratory and serological methods compared to other procedures in diagnostics, as well as the length of treatment, treatment outcomes and sequelae.

Methods

The study of WNV infection was conducted as retrospective study by analysing medical documentation of patients

who were treated at the Clinic for Infectious Diseases of the Clinical Centre of Vojvodina in the period from January 1, 2012 to December 31, 2013. The study included 32 patients diagnosed with neuroinvasive form of WNV infection based on clinical findings, laboratory analyses, and serological tests.

In all the patients we analysed clinical (febrile condition), laboratory indicators of inflammation process [sedimentation value, C-reactive protein (CRP) and leukocytes, liquor findings] depending on age and sex, on the day of admission and on the day 7 of hospital treatment.

We analysed the presence of febrile condition among patients at admission and on the day 7 of hospitalisation classifying them either as afebrile (body temperature up to 37°C), sub-febrile (body temperature from 37 to 38°C), or febrile (body temperature above 38°C). Lumbar puncture was performed at admission and on the day 7 of hospital treatment in all the patients within the analysed sample.

We monitored clinical and demographic indicators (patients' age, the presence of health problems on admission of patients to hospital treatment, duration of hospitalisation, place of residence).

Etiological diagnostics was carried out for all patients in the studied sample by means of isolation and identification of the virus and virus antigens from the patients' material using the method ELISA and/or by means of RT-PCR methods that were carried out at the Institute of Public Health of Vojvodina, Virology Department.

The results of the study were processed using standard statistical methods. The χ^2 and *t*-test were used for the purpose of statistical significance evaluation.

Results

Retrospective analysis showed that 32 patients with the diagnosed neuroinvasive form of WNV infection were hospitalised at the Clinic for Infectious Disease of the Clinical Center of Vojvodina in Novi Sad within a two-year period from the beginning of January 2012 to the end of December 2013. The distribution of patients by age showed the predominance of patients belonging to the working population [aged from 31 to 65 – 19 (59%)]. There were 3 (10%) pa-

tients younger than 30 and 10 (31%) geriatric patients (older than 65). Analysing the representation by gender among the observed patients we found 22 (69%) males and 10 (31%) females. The existence of statistically significant difference in distribution of patients by gender was not found, neither among the studied age groups ($p > 0.001$).

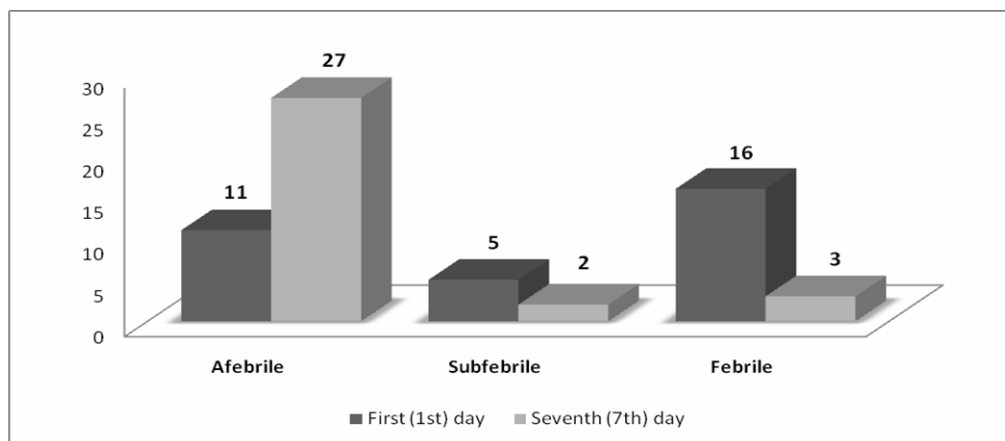
Febrile condition was present at admission in 21 (65.6%) patients, 5 (15.6%) were subfebrile and 16 (50%) febrile. On the day 7 of hospitalisation, we registered 27 (84.4%) afebrile, 2 (6.2%) subfebrile and 3 (9.4%) febrile patients. We presented the obtained results in Figure 1. Statistically significant difference was found between the number of febrile persons at admission and on the day 7 of hospital treatment ($p < 0.001$).

When it comes to indicators of inflammatory response, we analysed the CRP, total leukocytes count as well as lymphocytes values. In our sample there was no registered leukocytosis either at admission or on the day 7 of hospital treatment. Lymphocytosis was present in 2 (6.25%) cases at first as well as the day 7 of hospitalisation. The elevation of CRP at admission showed in 14 (43.75%) patients and in 12 (37.5%) patients on the day 7 of hospital treatment. There was no statistically significant difference between the analysed parameters ($p > 0.001$).

Etiological diagnostics was carried out at the Clinic for Infectious Diseases of the Clinical Center of Vojvodina by applying serological testing of patients. ELISA test was carried out for all hospitalised patients and all the findings were positive (100%). Out of a total of 32 patients, PCR was carried out in 10 (31.25%) of them. Positive PCR findings for the presence of WNV genome were found in 3 (30%) of the patients out of the total number of tests that were carried out.

At the moment of patients' admission, positive meningeal signs were present with the total share of 27 (84.4%). The frequency of individual symptoms within the meningeal syndrome at admission was registered as – headache (78.1%), vomiting (34.4%), neck rigidity (43.8%), and photophobia (6.25%).

Neurological signs were determined in 17 (53.2%) of the cases and consciousness disturbances in 10 (31.3%) cases. On the day 7 of hospital treatment, we registered the



$p < 0.001$

Fig. 1 – The presence of febrile condition at admission and on the day 7 of hospital treatment.

absence of consciousness disturbances and each individual symptom within the meningeal syndrome. The presence of neurological signs on the day 7 of hospitalisation was confirmed in 4 (12.5%) of the cases. We found a statistically significant difference between the share of the analysed clinical parameters (meningeal signs, neurological signs, consciousness disturbances) among individuals at admission and on the day 7 after the admission to hospital treatment ($p < 0.001$).

A percentage share of cerebrospinal fluid parameters representation is presented graphically in Figure 2.

We determined the presence of comorbidity and predisposing factors of the disease occurrence in the patients. Out of the total number of patients, the presence of accompanying diseases was registered in 12 (37.5%) of the cases. The highest frequency in the patients with comorbidity was exhibited by cardiovascular diseases (21.9%). We presented the distribution of the analysed groups of diseases in a graphic form (Figure 3) and found no statistically significant difference in the share of the analysed groups of diseases. The largest number of patients were released from the hospital treatment fully recovered, 28 (87.5%). Of the remaining 4 (12.5%) patients, 2 (6.25%) developed sequelae. Lethal outcome was registered in 1 (3.13%) of the cases.

Ciota et al.¹² confirmed that transmissive potential of *Culex pipiens* mosquito population decreased after WNV of the MP02 lineage infection despite high sustainability of those chains in the host's organism.

The development of WNV infection in endemic regions is linked with multiplication of population of *Culex* genus mosquitoes (*Culex pipiens*, *Culex restuans*, and *Culex tarsalis*) that are primary vectors. The above-mentioned mosquito genus pullulates and reproduces itself in stale, dirty, and putrid collections of ground water areas and flood prone regions, landfills and septic pits that are located within the residential infrastructure¹³.

Within the project titled "Detekcija virusa Zapadnog Nila kod populacije komaraca na teritoriji Republike Srbije" ("Detection of the West Nile virus among the mosquito population in the territory of the Republic of Serbia"), the Institute for Biocides and Medical Ecology from Belgrade performed the tests on mosquitoes in the territories of municipalities of Novi Sad and Temerin in September, 2013. The traps were set at potential sites and live mosquitoes of *Culex* genus were isolated under the professional supervision of doctors of veterinary medicine. They were transported in adequate conditions and subsequently tested by PCR method. Out of 10 sites in the

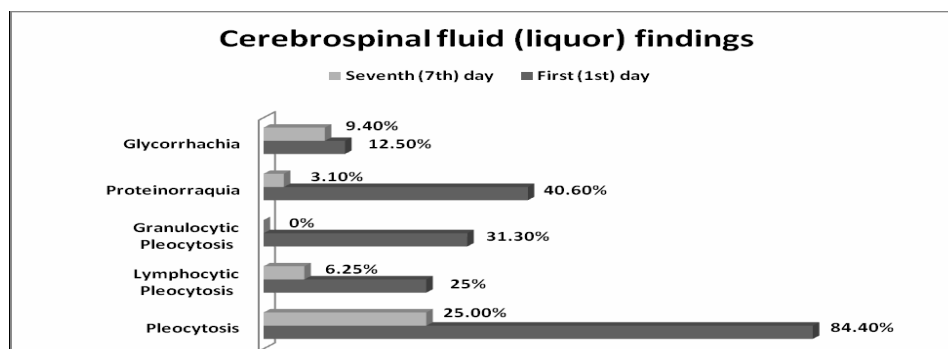


Fig. 2 – Registered values of the findings for cerebrospinal fluid (liquor).

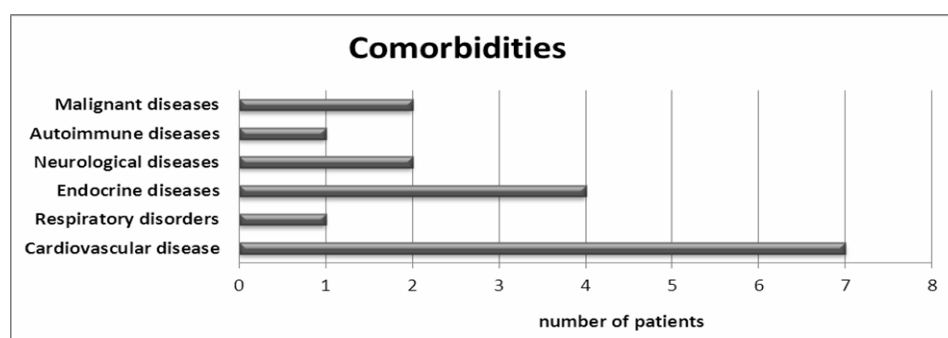


Fig. 3 – The share of registered groups of comorbidity among patients.

Discussion

Arboviruses, RNA viruses with huge inherent evolution potential, have been evolving very slowly until now. The life cycle and survival of the viruses of this group relies on vertical transmission that reduces their pathogen potential for the vertebrate host¹².

territory of Novi Sad, the presence of the viral genome was proved at 5 sites (50%). In the municipality of Temerin, the result of the PCR method was positive to the presence of the viral genome at one site out of 5 (20%) tested¹⁴.

In the sample of 32 patients who were treated at the Clinic for Infectious Diseases of the Clinical Center of Vojvodina during 2012 and 2013 and included in our study,

there were 15 (59.4%) of the patients with the residence in the territory of the municipality of Novi Sad and 9.4 (3/32) in the territory of municipality of Temerin. Our results are in compliance with the data obtained in the analysis of mosquitoes from the same territory.

Thanks to the data of the Institute for Public Health from Novi Sad and Institute for Biocides and Medical Ecology from Belgrade, we produced the map of the analysed sites in the territory of the municipality of Novi Sad and residence of citizens with positive laboratory findings.

The European and American researchers agreed that more than 90% of infections caused by arboviruses show seasonal character and occur in the period from July to September^{15, 16}. Both epidemic waves analysed in our study were recorded in the period from the beginning of August to the end of September 2012 and 2013.

In a long-term period from 1999 to 2012, more than 36,000 cases of WNV infection in human population were reported to the Centre for Disease Control and Prevention (CDC). Epidemiological department of this institution assess that starting from 1999 there were 2 to 4 million infected people in the territory of the USA, while some 400,000 to 1 million of infected people had some sort of symptomatology¹⁴. In the territory of the Republic of Serbia, there are no records on the number of infected people and the number of people with the West Nile virus.

In the Climatological Analysis of the Republic Hydro-meteorological Service of Serbia that was conducted by Smailagić et al.¹⁷, it was confirmed that the summer of 2012 was one of the hottest within the last few years. The obtained climatological data correlate with multiplication of the *Culex* genus mosquito population, and the occurrence of the first registered cases of infection with the West Nile virus in the territory of the Republic of Serbia.

The US CDC registered 5,245 cases of WNV infection until the end of November, 2012, including 256 lethal outcomes for the current year. Nasci¹⁸ states in his report that 51% of the total number of infected individuals have neuroinvasive form of the disease (meningitis/meningoencephalitis). This has been the highest annual incidence of WNV infection diseases in the USA since 2003.

Immediately after the WNV RNA lineages were isolated from the serum of goshawk in the territory of Hungary and after it was proven they circulated in the blood of the infection reservoir (birds, horses) in the territory of Hungary, Austria, and Italy, a sharp wave of epidemics among humans occurred in the territory of Greece in 2010. During two seasons, there were 273 registered cases of neuroinvasive forms of West Nile virus infection in Greece¹⁹.

In our region, all registered cases of WNV infection had neuroinvasive forms of the disease. The data from our study indicate the presence of neuroinvasive form of WNV infection (meningitis/meningoencephalitis) in all the patients within the analysed sample. It is assumed that the number of persons infected with WNV was much higher in both epidemic waves, during 2012 and 2013. Only those with the most severe forms of clinical picture were hospitalised and serologically tested.

Thanks to cooperation between the Clinic for Tropical and Infectious Diseases of the Clinical Center of Serbia and Institute for Virology Torlak, the report was compiled and the epidemic of WNV infection in the territory of Belgrade and its surroundings was evaluated in 2012. Within this study, Popović et al.³ emphasise the predominance of neuroinvasive forms of the disease. The results of our study are in compliance with the data from the study published by this group of authors from Belgrade.

The group of authors from Belgrade believe that the number of infected persons within the WNV epidemics in 2012 was higher than the registered one and that a significant number of patients with febrile condition resembling to influenza remained undiagnosed. It is assumed that these patients with nonspecific inflammatory symptomatology did not ask for medical assistance or they reported to the institutions of primary health care where they were treated symptomatically³.

The results of national studies are in compliance with the conclusions of wider professional public. Kwan et al.²⁰ determined in the study that encompassed a six-year period (2004–2010) that the number of reported cases of West Nile fever and neuroinvasive forms of WNV infection in the territory of the state of California was not a representational sample compared to the entire population. The above-mentioned authors indicate the reduced number of performed serological tests in persons with febrile condition, which reduces a total number of reported disease cases.

According to the estimates of the US CDC, 30 to 70 cases of moderate neuroinvasive forms of disease could be added to each reported case of neuroinvasive form of WNV infection¹⁵.

Epidemiological analysis of WNV infections in Texas was carried out in January 2013 for a multi-annual period from 2002 to 2011 and it was determined male Caucasian individuals aged 54 on the average dominate among the reported cases (59%)²¹. The data of the US CDC show insignificant differences in age and gender distribution in the territory of the USA – the share of male individuals among the ill makes 56% with the average age of 56¹⁵. Our results in the analysed sample indicate the predominance of male individuals (68.75%) with a high frequency of diseases among the working population aged 30 to 65 (59.38%), which is in compliance with the conclusions of the American authors.

Nolan et al.²¹ point to the mortality rate of 6.3% among patients with neuroinvasive WNV infection in the territory of Texas. The report from June 2013 describes that mortality or people with similar diseases in the entire territory of the USA reaches 5%, with the average age of 77 years¹⁵. Contrary to American studies, our study indicates a lower mortality rate among the patients – lethal outcome was found in 3.13% (1/32) of cases in the analysed sample.

PCR can be useful diagnostic procedure during an early stage of WNV infection. After seroconversion, it is much more likely that etiological diagnosis will be established *via* serological tests²². PCR testing was performed in 31.25% of patients in our study. A positive result was obtained in 30% of the analysed samples.

South African researchers Zaayman et al.²² from the University in Pretoria described a lower incidence of positive PCR results stating 2 factors that conditioned a small number of positive PCR WNV tests: 1) arbovirus viremia lasts for a short time and cerebrospinal fluid retains a small number of viruses, and 2) certain percentage of falsely negative results will occur if the samples are not stored properly. Turkish experts pointed out that they did not obtain a single positive result of RT-PCR to the presence of WNV RNA in the analysed sample²³.

Serological testing using the method ELISA was performed in all the patients who were included in our study, which enabled etiological diagnosing of the disease. The obtained results are in compliance with the data of the Clinic for Tropical and Infectious Diseases of the Clinical Centre of Serbia described by Popović et al.³ in their study. A larger number of European authors emphasise the significance and advantages of using the method ELISA for final diagnosing and determining of the causes of infection^{19, 22, 23}.

Garcial-Bocanegra et al.²⁴ believe that supervision over the domestic horses can be useful for monitoring of WNV genome circulation in endemic region, which is important from the aspect of public health. These attitudes are based on the established seroprevalence in horses who have not got ill and determined elevated frequency of neurological symptoms among horses compared to birds and humans²⁵.

Birds are the main natural WNV reservoirs. Local mobility of domestic birds and huge distances that migratory birds travel could contribute to spreading of the WNV infection²⁶⁻²⁹. Veterinary monitoring related to WNV infections was performed in the territory of the Republic of Serbia within the recent years. Petrović et al.⁷ from the Scientific Institute for Veterinary Medicine in Novi Sad performed epidemiological monitoring and testing over domestic birds and migratory birds from the territory of the AP Vojvodina in the period from January to September 2012. The WNV presence and circulation among birds in the territory of the Republic of Serbia was confirmed serologically for the first time in 7 (8%) of the analysed samples.

The preventive procedures aimed at the prevention of WNV infection occurrence imply public-health measures that will preclude mosquito bites in human population. The fact is that vaccines against domestic forms of arbovirus are not available. Forms of prevention of arbovirus infections rely on the activities of social community and personal efforts to reduce the vector population. Education of population on significance of prevention and methods of implementation of personal protection measures (*via* posters, leaflets, media campaigns) and implementation of system measures directed to the vector-mosquito population (destruction of habitats, larvae, and adult mosquitoes, use of insecticides) would enable combating new epidemic waves of WNV infection. The relevant bibliography states that the use of repellents correlates with the reduction of the risk of development of WNV infection^{3, 5, 17}. During the analysed epidemic wave in 2013, only the insignificant number of patients (3.13%) carried out personal protection measures and used repellents.

Conclusion

The absence of meningeal signs and fever on the day 7 of hospital treatment are indicators of good course and prognosis of neuroinvasive forms of WNV infection. The presence of comorbidities does not increase the risk of development of neuroinvasive forms of WNV infection. ELISA test is a sovereign diagnostic method compared to RT-PCR test that can serve as an indicator of acute infection. In most cases, after administering symptomatic therapy, the complete recovery of patients is achieved.

Acknowledgements

The presented work is a part of the research done in the project TR31084 granted by the Serbian Ministry of Education, Science and Technological Development.

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Received on July 18, 2014.

Revised on September 24, 2014.

Accepted on October 22, 2014.

Online First October, 2015.