SIGNIFICANCE OF IgG AVIDITY TEST IN DIAGNOSIS OF WEST NILE VIRUS INFECTION

ZNAČAJ TESTA AVIDITETA IgG ANTISETA U POSTAVLJANJU DJAGNOZE INFECIJE VIRUSOM ZAPADNOG NILA

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Summary

Introduction. Serological tests appear to be the method of choice for establishing the diagnosis in the late phase of West Nile virus infection. Long persistence of IgM antibodies against West Nile virus is described and may be a problem for determination of the time of acquisition of West Nile virus infection. The aim of the study was to estimate the significance of IgG avidity determination in establishing the diagnosis of West Nile virus infection. Material and Methods. In a study 56 serum samples seropositive against West Nile virus were included. 24 serum samples were collected in 2012 from healthy residents of South-Backa district and 32 serum samples were collected in 2014 from 124 patients suspected of having West Nile virus infection. Commercial enzyme-linked immunosorbent tests were used for the detection of West Nile virus-specific IgM and IgG antibodies and IgG avidity. Results. Out of 124 patients suspected of having West Nile virus infection, 32 (25.8%) were seropositive for West Nile virus antibodies. Acute infection was laboratory confirmed in 15 (46.9%) cases. All patients with acute infection were West Nile virus IgG positive, 13 (85%) were West Nile virus IgG positive, and 2 (15%) had a borderline result for West Nile virus IgG antibodies. Out of 32 seropositive patients the presence of IgM antibodies was determined in 22 (68.7%). In a group of samples with high IgG avidity values, 6 were IgM positive, while 8 were IgM negative. Conclusion. West Nile virus IgM and IgG antibody serological assays alone are not sufficient for the accurate and reliable diagnosis of WNV infection. West Nile virus IgG avidity testing is necessary to ensure the differential diagnosis of acute from past West Nile virus infection.

Key words: West Nile virus; West Nile Fever; Diagnosis; Immunoglobulin G; Immunoglobulin M; Antibody Affinity; Enzyme-Linked Immunosorbent Assay

Introduction

West Nile virus (WNV) is an arthropod-borne, neurotropic virus, with zoonotic potential, that belongs to the family Flaviviridae, genus Flavivirus.

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Japanese encephalitis serocomplex [1]. The members of this serocomplex are also other neuroviralnt viruses such as Murray Valley encephalitis virus, St. Louis encephalitis virus, or Usutu virus. WNV is maintained in nature in an enzootic transmission cycle between birds, that are the natural reservoirs of WNV, and ornithophilic mosquitoes that have a role of vectors. Humans are considered “dead-end” hosts for WNV, as the low level of viremia in mammals is usually not sufficient to be
transmitted to mosquitoes. WNV is an important human pathogen. It is estimated that 20% of infected people develop clinical symptoms and in less than 1% of them severe neurological diseases, meningitis, encephalitis, or acute flaccid paralysis, are observed [2]. For decades, the virus was endemic in Africa, southern Asia, north Australia and in many of the warmer regions of Europe. During the past several decades human WNV infections were mainly associated with sporadic cases, and since only sporadic outbreaks were reported, WNV was not considered a serious human health threat. WNV was introduced into New York in 1999, rapidly spreading to the entire country. The virus has become endemic in the United States, with thousands of human cases, and hundreds of neuroinvasive disease cases reported annually. Over subsequent years virus expanded to Canada, Mexico and Caribbean [3]. Endemic circulation of WNV has also been reported in many European countries. Great attention was paid to the epidemic of neuroinvasive cases of WNV human infections in Bucharest, Romania in 1996 when 393 cases of encephalitis and 17 deaths were recorded in people over 50 years old [4].

Although a serological study conducted in rural part of Vojvodina community revealed seroreactivity to WNV in up to 3% of the human population [5], the virus was not detected until 2010. The first direct detection of WNV in Serbia was from Culex pipiens mosquito pool collected in Novi Sad in 2010 [6]. Serological investigation of horse serum samples [7] and molecular investigation in wild birds [8] indicated that WNV circulated among animals in Vojvodina. The first outbreak with human cases of neuroinvasive WNV disease in Serbia was registered in 2012 when 58 patients were hospitalized at the Clinic for Infectious and Tropical Diseases, Clinical Centre Serbia in Belgrade. 52 of these patients had neuroinvasive and 6 had a febrile form of the disease. A total of 35 patients had completely recovered, while in nine patients fatal outcome was recorded [9]. Since 2012, human cases were recorded every year in Serbia. During 2013 and 2014, 32 patients were diagnosed with a neuroinvasive form of WNV infection and were treated at the Clinic for Infectious Diseases of the Clinical Centre of Vojvodina. Full recovery was recorded in 87.5% and lethal outcome in 3.13% of them [10].

Several different tests have been developed for the laboratory diagnosis of WNV infections. In the early phase of illness, diagnosis is based on the detection of viral ribonucleic acid (RNA) by reverse transcription polymerase chain reaction (RT-PCR) assays, real-time RT-PCR, and nucleic acid sequenced-based amplification. Identification of the WNV RNA in the CSF or serum during the acute stage of neurological involvement is generally considered to be a confirmatory diagnostic parameter. Viral isolation from tissues, blood or cerebrospinal fluid on cell culture (such as Vero E6, RK-13, AP61 or C6/36) is usually unsuccessful even in the early stage of infection because of the low viral load in humans. Virus isolation must be performed under biosafety level 3 conditions (BSL 3).

However, serological tests appear to be the method of choice for establishing the diagnosis in the late phase of virus infection. Significant problems in serological diagnosis are cross reactions between members of Flavivirus genus due to antigenic similarity [11]. Vaccination against Japanese encephalitis virus (JEV) or Yellow fever virus (YFV) can yield false positive results in enzyme-linked immunosorbent assays (ELISA) (ELISA, Euroimmun, Luebeck, Germany) IgM test for WNV [12]. Long persistence of IgM antibodies against WNV is described [13], and may be a problem for determination of the time of acquisition of WNV infection. Avidity IgG test can be used to help distinguish a recently acquired from past WNV infection [14]. Avidity is the binding intensity of interactions between the antibody and antigen. At the beginning of infection antibodies of low avidity are produced. During infection, in the process of affinity maturation, the avidity of IgG antibodies increases progressively there after within few months (high avidity) [15]. In the aim of determination of the avidity, the same sample is tested twice, applying ELISA or indirect immunofluorescence test. Once the sample is treated with urea as denaturing factor and parallel sample is exposed to phosphate buffer. In the process of denaturation low avidity IgG antibodies will liberate from antigen and high avidity antibodies will remain attached [16].

The aim of the study was to estimate the significance of IgG avidity determination in establishing the diagnosis of WNV infection.

**Material and Methods**

Between June and December of 2014, in the Centre of Virology at the Institute of Public Health of Vojvodina, blood serum samples from 124 patients suspected of having WNV infection were tested for the presence of antibodies against WNV. In addition, 24 serum samples collected before June 2012, from healthy residents of South-Backa district who were referred to the Institute of Public Health of Vojvodina for preoperative examination and were WNV IgG positive, were also included in the study. Commercial ELISA were used for the detection of WNV-specific IgM and IgG antibodies. Testing,
calculation, and interpretation of the results were performed strictly following the instructions of the manufacturer. Results were evaluated semiquantitatively by calculating a ratio of the extinction value (optical density value - O.D.) of the patient sample over the extinction value of calibrator 2 which was included in the test. Results were considered as positive if the ratio was equal to or greater than 1.1, borderline if the ratio was between 0.8 and 1.1 and negative if the ratio was less than 0.8. For determination of IgG avidity commercial ELISA using urea as a denaturing factor was carried out as described by the manufacturer. Serum samples were tested in duplicate: in one well with phosphate buffer and in the other with urea treatment. A relative avidity index (RAI) was calculated and expressed as a percentage by dividing the OD values with and without urea treatment and interpreted as follows: <40% low RAI indicating acute infection; 40–60% borderline RAI indicating recent infection; > 60% high RAI indicating past WNV infection.

**Results**

Out of 124 patients suspected of having WNV infection, 32 (25.8%) were seropositive for WNV antibodies. Acute infection was laboratory confirmed in 15 (46.9%) cases, 3 (9.4%) patients had a recent infection, and 14 (43.7%) patients had past WNV infection. Results of serological testing and IgG avidity testing for serum samples of patients with acute or recent WNV infection are presented in **Table 1**. Blood samples were obtained from 12 patients 5 to 37 days after onset of infection, while for 6 patients the data regarding the duration of illness were not available. All acutely ill patients were IgM positive and had IgG antibodies of low avidity (RAI<40%). Avidity index values were in the range from 15.1% to 38.7%. Two patients with acute infection had borderline results for IgG antibodies in ELISA (0.93 and 1.02) and avidity testing showed low values of RAI (32.7 and 22.7). Only in 7 (21.9%) cases, acute infection was confirmed by seroconversion between acute and convalescent serum sample. Borderline avidity results indicating recently acquired WNV infection were observed in three patients. Among them, 1 had borderline (0.93) and 1 had a negative result for IgM antibodies. In a group of patients with no available information about the date of symptom onset, there were 3 cases of acute infection and 3 cases of recent infection indicated by RAI values between 40% and 60%.

Nine (28.1%) patients were IgM negative and one had borderline IgM result (0.93). Positive re-

<table>
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<th>Relative Avidity Index of IgG antibodies (%)</th>
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sults for IgG antibodies were obtained in 27 (84.4%) cases, while 5 (15.6%) patients had borderline IgG results. As Graph 1 shows, all low IgG avidity serum samples were IgM positive. Among serum samples with borderline IgG avidity values, there was one positive, one negative, and one with borderline IgM result in ELISA. In a group of samples with high IgG avidity values, 6 were IgM positive, while 8 were IgM negative.

Results of IgG avidity test for serum samples of 24 healthy persons collected before the WNV epidemic 2012 are presented in Graph 2. Acute infection was confirmed in 1 (4.2%) case according to positive results for IgM and IgG antibodies in ELISA and low RAI (26%). Borderline values of RAI (49% and 55%) suggesting recent infection were found in 2 (8%) cases. The most seropositive healthy persons (22/25, 88%) had high RAI values, ranged from 68.4% to 98%, which was considered indicative of past exposure to WNV.

Values of RAI for 12 acutely ill patients with available data about the duration of illness are shown in Graph 3. Low avidity IgG antibodies were detected on the 5th day of illness and have (been) demonstrated to be a powerful tool for early diagnosis of WNV infection. However, due to the short period of viremia molecular diagnostics is reliable only within the first few days of illness. Results of a study done by Busch et al. [17] indicated that WNV RNK was detectable, on the average, until 13.2 days post infection. Isolation of virus in cell cultures has the similarly limited significance in the diagnosis of WNV infection. It is useful only at the beginning of the infection. Moreover, isolation of WNV is time-consuming and requires the use of cell cultures and live, infectious virus so it must be performed under biosafety level 3 conditions which make it more cumbersome than the detection of WNV RNA by molecular assays.

Molecular and culture-based methods are applicable if the sample can be collected in the beginning of the infection, while WNV specific antibody detection in serum or liquor samples is the best approach for the diagnosis of WNV infection in later stages of illness. The most widely used serological method is ELISA because it is sensitive, easy to perform and can be fully automated which enables rapid simultaneous analysis of a large number of samples. Using ELISA, Prince et al. [18] discovered that WNV IgM and IgA antibodies can be detected 3 to 9 days and IgG antibodies 4 to 16 days, after the initial positive result for WNV RNK in blood. In our study, WNV IgM and IgG antibodies were determined in 22 (68.7%) patients suspected of having WNV infection and tested within 37 days from the onset of illness. However, IgG avidity testing confirmed acute infec-
tion in only 15 (46.9%) of them, while high IgG avidity test results indicated past infection in 6 (27.3%) cases. Diagnosis of past WNV infection was also established according to high IgG avidity test results (RAI >60%) in 8 WNV IgM negative patients. These results suggest that positive WNV IgM result is not always a proof of acute infection, which makes the diagnosis of WNV infection very difficult. The main reason for that is prolonged IgM response in WNV infected persons. Long-term persistence of WNV IgM antibodies, commonly for many months, is documented in a number of studies. Papa et al. detected low levels of WNV IgM antibodies in 3 out of 10 patients, 3 years after primary infection [13]. The presence of high avidity IgG antibodies in those 3 patients confirmed past infection. Busch et al. followed up 245 WNV RNK positive blood donors in order to assess WNV antibody development and persistence [17]. The mean time from the detection of WNV RNK to the detection of WNV antibodies in serum samples was 3.9 days for IgM and 77 days for IgG class. The mean time of antibody persistence was 156 days for IgM and 220 days for IgA antibodies. In a study of Roehrig et al., WNV IgM antibodies were determined in serum samples from 7 of 12 patients at approximately 500 days after onset of acute WNV encephalitis [19]. Results of an investigation done by Prince et al. indicated that specific IgM antibodies were detectable in 17%, IgA antibodies in 57% and IgG antibodies in 100% of 23 blood donors one year after the primary WNV infection [20]. Murray et al. also proved long-term persistence of WNV IgM antibodies. They confirmed the presence of WNV IgM antibodies 1, 6 and 8 years following acute infection in 42%, 34%, and 23% of patients, respectively [21]. Antibodies of IgM class do not cross the blood-brain barrier, so the presence of WNV IgM antibodies in cerebrospinal fluid strongly suggests acute central nervous system infection. However, there is some evidence of WNV IgM antibody persistence in cerebrospinal fluid from patients with neuroinvasive WNV infection, 110, 144 and 199 days after the onset of illness [22]. In addition to WNV IgM antibody persistence, data from animal models and patients who recovered from WNV encephalitis suggest that WNV not only causes acute disease but can also cause persistent infection. Prolonged excretion of WNV with urine (1.6 to 6.7 years) indicates replication of the virus in kidneys and persistent renal infection [23].

In our investigation, acute WNV infection was confirmed in 15 (46.9%) patients, while in 3 (9.4%) cases recent infection was demonstrated. All patients with acute infection were WNV IgM positive, 13 (85%) were WNV IgG positive, and 2 (15%) had a borderline result for WNV IgG antibodies. In all acutely ill patients, WNV IgG antibodies of low avidity were found with a RAI ranging from 15.1% to 37.8%. In 7 cases current infection was confirmed by the WNV IgM antibody detection in the acute-phase sample with seroconversion to IgG in a convalescent-phase sample. Documentation of seroconversion has been the “gold standard” for diagnosis of acute WNV infection. Taking into account that convalescent sample is not always available, IgG avidity testing is a useful tool for distinguishing current from recent or past infection in those cases.

Determination of the avidity of IgG antibodies may provide useful information regarding the timing of infection allowing differential diagnosis of acute from recurrent or past infection [24]. The IgG avidity test has been successfully used for the diagnosis of acute infections during pregnancy caused by teratogenic pathogens including protozoan parasite Toxoplasma gondii [25] and cytomegalovirus [26]. It has also been applied in the diagnosis of infections with flaviviruses such as WNV [27], tick-borne encephalitis virus (TBEV) [28] and Dengue viruses [29].

In this investigation, WNV IgM antibodies were determined in 7 of 25 (28%) WNV IgG positive healthy persons. Most of them (84%) had WNV IgG antibodies of high avidity. These findings are in agreement with other studies which have also documented WNV IgM antibody persistence over an extended period of time, indicating that WNV IgM antibodies are not a reliable marker of acute infection. It is estimated that approximately 80% of WNV infections are subclinical or asymptomatic [2]. In this study, acute asymptomatic infection was confirmed in 1 person with no clinical signs of illness.

Another problem that limits the clinical relevance of serological assays in WNV diagnostics is a high degree of cross-reactivity of antibodies produced in response to infection with different members of genus Flavivirus including St. Louis encephalitis virus, JEV, JFV, and Dengue 1–4 viruses. Antigenic similarity of the flavivirus envelope glycoprotein results in eliciting a cross-reactive antibody response and occurrence of false-positive results in serological assays [30]. A problem with misinterpretation of serological results is particularly prominent in geographic regions with co-circulation of various flaviviruses and implemented programs for vaccination against TBEV, YFV and/or JEV. The issue of cross-reactivity may be overcome by performing the plaque reduction neutralization test (PRNT) which has a high degree of specificity for target flaviviruses [31]. However, PRNT must be carried out under the biosafety level 3 conditions as a viable virus is used in this assay, and these facilities are available only in specialized research institutions not in routine diagnostic virology laboratories. Lack of PRNT as confirmatory assay was the limitation of this study. Immunofluorescence-based assays, such as “Flavivirus Profile 2” (Euroimmun, Luebeck, Germany) may be helpful in distinguishing infections caused by TBEV, WNV, JEV, YFV, and Dengue 1–4 viruses [32]. Recently, a new improved serological assay for discrimination of infections with WNV from those with other flaviviruses has been developed. It is based on differences in binding affinity to recombinant mutant E protein between WNV and other flaviviruses. An-
tibodies against WNV bound equally well to the wild type and the mutant type of protein, while antibodies from persons infected with TBEV and JEV show decreased affinity to mutant E protein [30].

Conclusion

The findings from a number of studies on West Nile virus IgM antibody persistence imply that serum IgM antibodies should not be considered a marker of acute West Nile virus infection. The results from our investigation concerning the detection of West Nile virus IgM antibodies in healthy persons as well as in patients with high avidity West Nile virus IgG antibodies, also suggest that the presence of West Nile virus IgM antibodies in serum samples do not necessarily indicate acute infection. Therefore, West Nile virus IgM and IgG antibody serological assays alone are not sufficient for the accurate and reliable diagnosis of West Nile virus infection. West Nile virus IgG avidity testing is necessary to ensure the differential diagnosis of acute from past West Nile virus infection.

References