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4. RESULTS OF THE INVESTIGATION OF WEST NILE VIRUS ACTIVITY IN HORSES IN SOME PARTS OF SERBIA

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Abstract

West Nile virus (WNV) is the most widely spread Flavivirus around the world. Its transmission cycle involves mosquitoes and birds, but human and horses are also susceptible to the infection. WNV causes a high morbidity and mortality in birds and, to a lower extent, in horses and human. The virus has been responsible for occasional outbreaks in Europe, Africa, the Middle East and some parts of Asia for many years, but since 1990’s the number, frequency, and severity of cases has increased. In fact, in the past few years an increasing number of severe outbreaks in horses have been reported in Italy, Romania and more recently in Spain.

Since the risk WNV spread throughout the continent is worrisome for animal and human health, we have conducted, for the first time in Serbia, a serological survey for the presence of anti-WNV antibodies in healthy horses. A total of 349 healthy horse’s sera randomly collected during 2010 in Belgrade district area, the municipality of Sabac, and 26 municipalities in Vojvodina province were tested for the presence of anti-WNV IgG by a validated in house ELISA based on the use of a baculovirus expressed recombinant E viral glycoprotein partially purified from infected insect larvae extracts and by plaque reduction neutralization test (PRNT). Our results showed that 42/349 (12%) of the horse’s sera presented anti-WNV IgG neutralizing antibodies. Average PRNT and ELISA titers were 140 and 10.5, respectively.

A further study was conducted in 255 stored serum samples collected from healthy horses in 7 different locations in Vojvodina and Belgrade region during 2007 to 2011. The samples were tested by an immunocassay blockage commercial ELISA kit (INGEZIM West Nile COMPAC, Ingenasa, Spain). Of the 255 samples, 72 (29%) resulted positive. When retested by the in house ELISA, 90% concordance was found. Likewise, all 48 IgG positive samples also resulted positive by PRNT.

According to these data, we can conclude that West Nile virus is present and circulating in Serbian horse population. Although no clinical signs of illness were

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reported in horses and human yet, the epidemiological situation in surrounding countries warn us that an extensive investigation is necessary to conduct in our country.

**Key words:** West Nile virus, horses, seroprevalence, ELISA, PRNT

**Introduction:**

West Nile virus was for the first time isolated in 1937 in Uganda. Since then, the virus is responsible for thousands of disease cases, often with the fatal outcome, in birds, horses and humans. WNV belongs to the family Flaviviridae (genus Flavivirus), together with other pathogens such are: dengue, yellow fever and Japanese encephalitis viruses. The virus posses the positive -strained RNA, has icosahedral capsid structure and the size of approximately 50 nm. Currently, phylogenetic analyses discovered 5 lineages of WNV. The virus which entered to North America belongs to lineage I (strain 1a). The same lineage is found in Europe, the Middle East and Africa. The Australian strain of WNV belongs to lineage Ib, and lineage II is discovered in Africa. There are still little information about lineage III, IV and V (Rossi et al., 2010).

The transmission cycle in nature involves mosquitoes and birds, but human and horses are also susceptible to the infection. The high percentage of morbidity and mortality is recorded in birds and with the lower extent in horses and human. The WNV causes occasionally outbreaks of the disease in Europe, the Middle East and some parts of Asia. Human cases of infection were usually asymptomatic with the clinical signs of mild fever, but approximately 1% of infected persons had encephalitis and meningoencephalitis, fever, headache, nausea and vomiting. Rarely, the virus caused death. In horses, WNV is often clinically unapparent, but 10% of cases develop neurological disorders with up to 50% mortality rates (Castillo-Olivares et al., 2004; Blitvich 2008; Calistri et al., 2010). Since 1990's the epidemiological situation changed and the number and severity of the virus virulence changed and caused dozens of human and horses deaths in Romania, Russia and the Mediterranean basin. In North America, in the region of New York State, the WNV infected about 2.6% of the residents. Since then, it has already caused over 1,100 human fatalities and over 12,000 cases of meningitis/encephalitis (Murray et al, 2010). In the recent years human WNV disease is reported in: Morocco in 1996, Tunisia in 1997, the Czech Republic in 1997, France in 2003. The enzootic that involved horses were reported in many countries, such are: Canada, France, Hungary, Croatia, Cuba, Morocco, Senegal, Israel, etc. (Rossi et al., 2010). The neurological disorders and deaths in horses were reported in Italy, Romania and Russia. Recreantly, the new outbreaks of WNV infection in human with 27 deaths has been reported during the year 2010 in Greece and neighboring countries (http://www.ecdc.europa.eu). Along with the human cases, there were the increasing numbers of severe outbreaks in horses. In Italy in 2008, 251 stable were infected, with 794 cases and 5 deaths (Calistri et al). In 2010 the first outbreak of WNV infection in horses has been reported in Spain, with 102 clinically ill cases and 15 fatal deaths (Garcia-Bocanegra et al., 2011).
Material and methods and results:

Since the risk of spreading of WNV through the continent is high, the aim of our study was to analyze, for the first time in Serbia, the presence of specific anti-WNV antibodies in healthy horses. We have randomly collected 349 horse sera during 2009-2010 in the Belgrade district area, in the municipality of Sabac and in 26 municipalities in Vojvodina province in the northern part of Serbia. Of the total number of sera, almost half of the animals (48.4%) were racing horses, 36% Lipizzaner breed horses, 10.2% ponies, 1.7% Arabian horses and 3.8% were mixed breed horses. Mean age of the animals were 7, 9 years (range 3 to 19). There were 59, 3% of mares and 40, 7% of stallions. The animals didn’t have any clinical signs of neurological disorders.

The sera were tested for the presence of anti-WNV IgG antibodies by a validated in house ELISA test. The principle of the test is based on the use of baculovirus expressed recombinant E viral glycoprotein partially purified from infected insect larvae extracts, that is explain elsewhere (Alonso-Padilla et al., 2010). Plaque reduction neutralization test (PRNT) was performed under bio-safety level 3 conditions on Vero cells.

Our results showed that 42/349 (12%) of the tested sera were IgG positive. All 42 sera neutralized WNV activity, but with the exception of one serum, none of them neutralized USUV. Also, none of 30 randomly selected sera were PRNT positive for any tested virus. The results of virus isolation on cell culture and real-time RT-PCR in any of the analyzed sera were also negative. The procedure of the assays were described elsewhere (Lanciotti et al., 2000) Average PRNT and ELISA titers were 140 and 10.5, respectively. Two of the positive horses were imported from Hungary and Croatia. There were no significant differences between positive animals regarding gender (mare 13%, stallions 9.8%). Seropositive animals were found in 14 of 28 studied municipalities (figure 1).

![Figure 1](image-url)

**Figure 1:** left: WNV positive countries (empty squares- humans, horse-black circles); right: tested WNV municipalities in Serbia (in grey); positive WNV municipalities (in black)

A further survey was conducted in 255 stored horse sera samples. All the sera were collected from 7 different locations in Vojvodina province and Belgrade, during 2007-2011. Horses included in this survey were of different breeds and different age.
The samples were collected from stables. None of the tested horses showed clinical signs of the WNV disease.

The presence of specific anti-WNV antibodies were tested by immunoassay blockage commercial ELISA kit (Ingezim West Nile Compack, Ingenasa, Spain). Of the 255 samples, 72 (28.6%) resulted seropositive. Conformation of seropositive samples has been performed by PRNT. Some of the samples were collected in very small quantities, so the PRNT test was performed on 48 samples that were positive in commercial ELISA test (data not published yet). The distribution of seroprevalence among horse in stables is presented in Table 1:

Table 1: Number of tested and positive WNV horses

<table>
<thead>
<tr>
<th>Location</th>
<th>Number of tested</th>
<th>Number of positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bečej</td>
<td>32</td>
<td>7</td>
<td>21.9</td>
</tr>
<tr>
<td>Kelebijja</td>
<td>36</td>
<td>12</td>
<td>33.3</td>
</tr>
<tr>
<td>Karadjordjevo</td>
<td>21</td>
<td>3</td>
<td>14.3</td>
</tr>
<tr>
<td>Surduk</td>
<td>30</td>
<td>4</td>
<td>13.3</td>
</tr>
<tr>
<td>Pančevo</td>
<td>45</td>
<td>16</td>
<td>35.5</td>
</tr>
<tr>
<td>Vršac</td>
<td>45</td>
<td>18</td>
<td>40.0</td>
</tr>
<tr>
<td>Beograd</td>
<td>43</td>
<td>12</td>
<td>27.9</td>
</tr>
<tr>
<td><strong>Σ</strong></td>
<td><strong>252</strong></td>
<td><strong>72</strong></td>
<td><strong>28.6</strong></td>
</tr>
</tbody>
</table>

The highest percentage of seropositive samples has been recorded within the horse population in Vršac and the lowest seroprevalences to WNV have been found in Surduk and Karadjordjevo regions.

Discussion:

In the first investigation that we have conducted, the results showed that 12% of tested horse sera presented specific neutralizing antibodies against WNV. From the former territory of Yugoslavia, only available data about WNV activity are from 1970-1980's, where the authors described 0.5-7.9% seroprevalence among inhabitants (Vesnjak-Hirjan et al., 1991) and a study reporting a low seroprevalence of 0.4% in tested horse sera in Djakovo region in Croatia (Madic et al., 2007). According to these data, we can conclude that WNV is circulating in the region. Also, one horse presented PRNT90 titer against USUV and WNV. The presence of USUV was reported in Europe in birds (Weissenbock et al., 2002). To our knowledge, this is the first time that USUV neutralizing antibodies are described in horses.

In the second research, we also proved that WNV is circulating in the region and 28.6% of tested horses sera were WNV seropositive. The highest percentage of positive horses was found in Vršac, the region which is close to Romanian border. In Romania the WNV outbreak in horses and humans were recorded in relative close region. The high level of 26.9% of neutralizing anti-WNV antibodies in horses comparing to 12% reported in our first investigation could be also explained by the fact
that in the first study the horse sera were collected randomly, and in the second study the blood sera were taken from horses situated in the stables. Also, in two horses groups in the second survey (Surduk and Karadjordjevo) the lower level of seropositivity was described, comparing to other regions. These animals were kept most of the time in boxes, without outdoor contacts and probably were less exposed to the contact with the mosquitoes.

Although so far there were no reported cases of clinical disease in horses or even humans, the epidemiological situation in the neighboring countries over the past few years, warns us that it is necessary to conduct extensive tests in our country, as well as the possibility of implementing a special program of surveillance and disease control.

Acknowledgments:

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References:


РЕЗУЛЬТАТИ ИСТРАЖИВАЊА АКТИВНОСТИ WEST NILE ВИРУСА КОД КОЊА НА НЕКИМ ПОДРУЧЈИМА У СРБИЈИ

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Кратак садржај

West Nile вирус (WNV) је најраспространенији Flavivirus у свету. У природи вирус циркулише између комараца и птица, али људи и коњи су такође подложни инфекцији. WNV изазива висок морбидитет и морtalитет код птица, а у мањем броју случајева, код људи и коња. Вирус је одговоран за повремене случајеве избијања инфекције у Европи, Африци, Средњем Истоку и неким деловима Азије током дужег временског периода, али од 1990-их година број, учесталост и тежина болести се повећала. Такође, у последњих неколико година забележен је повећан број избијања тежих облика инфекције код коња у Италији, Румунији а и Шпанији.

Будући да је ризик од ширења инфекције узрокован West Nile вируsom на континенту забринувајучи, посебно за здравље животиња и људи, извршили смо серолошко испитивање присуства антитела против WNV код здравих коња. Укупно је тестирано 349 серума здравих коња на присуство анти-WNV IgG антитела. Узорци су сакупљени методом случајног избора током 2010. године у области Београда, са територије општине Шабац и 26 општине у Војводини. Тестирање је извршено валидованом in house ЕЛИСА методом заснованом на коришћењу делимично прежиђеног рекомбинантног вирусног гликопротеина Е из екстракта инфицираних ларви инсеката, добијеном методом експресије преко вируса инсеката (baculovirus) као и неутрализационим тестом редукције плакова (PRNT). Наши резултати су показали да 42/349 (12%) коњских серума поседује IgG неутрализациона антитела против WNV. Просечне вредности титра PRNT и ЕЛИСА теста износиле су 140 и 10,5.

Следеће испитивање је спроведено на 255 серума коња из колекције узораца сакупљених током 2007-2011. године на 7 различитих локација у Војводини и територији општине Београд. Узорци су били тестирани blockiнг ЕЛИСА комерцијалним тест китом (INZEZIM West Nile COMPAC, Ingenasa, Шпанија ). Од прегледаних 255 узораца, 72 (29%) је било позитивно. Узорци су
помено тестирани *in house* ЕЛИСА тестом, а резултати испитивања су у 90% испитаних серума били идентични. Такође, свих 48 IgG позитивних узора били су позитивни и приликом тестирања *PRNT* тестом.

Наши резултати показују да је *West Nile* вирус присутан и ширкулише у популацији коња у Србији. Иако, колико је нама познато, до сада нема лабораториски потврђених клиничких облика болести ни код коња нити код људи, епидемиолошка ситуација у земљама у окружењу у последњих пар година упозорава нас да је потребно спровести опсежна испитивања у нашој земљи, као и на могућност имплементације посебног програма надзора и контроле болести.

**Кључне речи:** *West Nile* вирус, коњи, сропреваленца, ЕЛИСА, *PRNT*