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WEST NILE VIRUS IN HORSES IN HUNGARY: EPIDEMIOLOGY, DIAGNOSTIC AND PREVENTION TECHNICS

Ph.D. THESIS

WRITTEN BY:

DR. ORSOLYA ESZTER FEHÉR

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University of Veterinary Medicine, Budapest

Doctoral School of Veterinary Science

Supervisor:

.....

Dr. Orsolya Korbacska-Kutasi PhD, Dipl. ECEIM

Associate Professor

University of Veterinary Medicine Budapest

Lectors:

.....

Dr. Zoltán Bakos PhD, Dipl. ECEIM

Associate Professor

University of Veterinary Medicine Budapest

.....

Dr. Győző László Kaján PhD

Senior research fellow

Institute for Veterinary Medical Research, Centre for Agricultural Research, Budapest

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Dr. Orsolya Eszter Fehér

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Abbreviations

BSL	–	Biosafety Level
CNS	–	Central Nervous System
CSF	–	Cerebrospinal Fluid
ECDC	–	European Centre for Disease Prevention and Control
EHM	–	Equine Herpesvirus Myeloencephalopathy
ELISA	–	Enzyme-linked immunosorbent assay
HIT	–	Hemagglutination Inhibition Test
HRV	–	Heart Rate Variability
IFA	–	Immunofluorescence Assays
IgG	–	Immunoglobulin G
IgM	–	Immunoglobulin M
IHC	–	Immunohistochemistry
JEV	–	Japan Encephalitis Virus Complex
LIV	–	Louping Ill
MIA	–	Microsphere Immunoassay
NÉBIH	–	Nemzeti Élelmiszerlánc-biztonsági Hivatal (Hungarian National Food Chain Safety Office)
NNK	–	Nemzeti Népegészségügyi Központ (National Public Health Center)
PCR	–	Polymerase Chain Reaction
PRNT	–	Plaque Reduction Neutralizing Test
RT-PCR	–	Reverse Transcriptase-polymerase Chain Reaction
SN	–	Serum-Neutralizing
TBEV	–	Tick-Borne Encephalitis Virus
USUV	–	Usutu Virus
VNT	–	Virus Neutralizing Tests
WNE	–	West Nile Encephalitis
WNF	–	West Nile Fever
WNM	–	West Nile Meningitis
WNP	–	West Nile Poliomyelitis
WNND	–	West Nile neuroinvasive disease
WNV	–	West Nile Virus
WOAH	–	World Organization for Animal Heath
YF	–	Yellow Fever

Summary

West Nile virus (WNV) is an emerging pathogen in Hungary, causing severe outbreaks in equines and humans since 2007. Mosquitoes transmit this zoonotic arbovirus between wild birds (natural hosts) and other vertebrates. Horses and humans are incidental, dead-end hosts but can develop severe neurological disorders. In the last decade in Hungary and the neighboring countries, West Nile neuroinvasive disease (WNND) has been caused in dramatically increasing numbers by lineage 2 West Nile virus strains both in horses and in humans. The disease in this geographical region is seasonal, so the vaccination of horses should be carefully scheduled to maintain the highest antibody titers during outbreak periods.

Our first study aimed to provide a comprehensive report on the clinical signs of WNND in horses in Hungary. Clinical details of 124 confirmed equine WNND cases were collected between 2007 and 2019. Data about the seasonal and geographical presentation, demographic data, clinical signs, treatment protocols, and disease progression were evaluated. Starting from an initial case originating from the area of possible virus introduction by migratory birds, the whole country became endemic to WNV over the next 12 years. Transmission season has not expanded significantly during the data collection, but vaccination protocols should continually be reviewed according to recent observations. There was no considerable relationship between the occurrence of WNND and age, breed, or gender. Ataxia was the most common neurological sign related to the disease, but weakness, behavioral changes, and muscle fasciculation appeared frequently. Apart from recumbency combined with inappetence, no other clinical sign or treatment regime correlated with survival. The survival rate showed a moderate increase throughout the years, possibly to increased awareness of practitioners.

Our objective in the second study was to compare the findings from the cerebrospinal fluid (CSF) samples of horses with WNND with those of healthy controls. Owing to the close contact of CSF with the extracellular fluid of the brain, the analysis of CSF composition can reflect central nervous system (CNS) impairments enabling the diagnosis and understanding of various neurodegenerative CNS disorders. We compared findings from fifteen CSF samples of 13 horses with acute WNV encephalomyelitis with 20 healthy controls. Protein, particular enzymes, ions, glucose, and lactate showed abnormal levels in a significant number of WNV cases. None of the six horses with elevated glucose concentrations survived. There was more neutrophilic than mononuclear pleocytosis identified with WNV infection. Neutrophils probably play a role in developing inflammatory responses and brain damage. Although elevated

glucose levels reliably predicted the outcome, they might be the consequence of increased plasma levels and reflect general stress rather than CNS pathophysiology. The CSF findings of WNV encephalomyelitis patients are non-specific and variable but facilitate the differential diagnosis.

In the third study, we summarize the clinical diagnostic and treatment features of WNND specific to Hungary and describe two cases of WNV neurologic disease with a particular focus on how recumbent neurological cases could be managed in stable conditions. Case management and clinical examination were performed at the home premises of both patients. The diagnosis in both cases was established according to the OIE guidelines based on the seasonality, clinical signs, and IgM ELISA serological positivity of acute infection. One horse needed intermittent assistance to rise, while the other became chronically recumbent during the acute phase of the illness. In the second case, a homemade sling was designed to support the patient in a standing position. In both cases, treatment was successful, and both horses recovered from the disease. Although the vaccine is available for the protection of horses, its high price and the lack of general awareness result in weak protection on a population level. We draw the attention of all veterinarians to the fact that WNV neuroinvasive disease is a real threat to the Hungarian equine population. We also give some ideas and guidelines on how patients could be managed in their home premises on a low budget when clinical admission is not possible.

The aim of our fourth study was to characterize the serum-neutralizing (SN) antibody titers against a lineage 2 WNV strain in response to vaccination with an inactivated lineage 1 vaccine (Equip® WNV). Thirty-two seronegative horses were enrolled in the study, 22 were allocated to the vaccinated group, and 10 were retained as unvaccinated controls. Horses were vaccinated according to the product's vaccination guidelines. Primary vaccination of two doses administered 28 days apart was initiated approximately 5 months before the WNV outbreak season, followed by a booster vaccination one year later. Blood samples were collected during a 2-year period to monitor the production of SN antibodies against lineage 1 and the enzootic lineage 2 WNV strain. Mean antibody titers against lineage 1 WNV were significantly higher ($P \leq 0.05$) in the vaccinated group compared to the control group at all-time points after the primary vaccination dose. SN antibody titers appeared significantly higher against lineage 1 than lineage 2 at all-time points. Similarly, mean antibody titers against lineage 2 WNV were significantly higher ($P \leq 0.05$) in the vaccinated group compared to the control group at all time points except at 6 months after the primary vaccination. According to the results, vaccination with an inactivated lineage 1 vaccine induces the production of antibodies against both WNV lineages 1 and 2 strains up to 2 years after booster vaccination. However, in those

geographical regions where lineage 2 strains are responsible for seasonal outbreaks, a booster vaccination should be considered earlier than 12 months after primary vaccination.

During the completion of this Ph.D. study, we published its partial results several times at Hungarian and international conferences, as well as the completed research sections in well-known international scientific journals.

Introduction

West Nile virus (WNV) is getting a more prominent threat every year in Europe. Hungary reports cases of more species every year since the country's first virus detection. Equines play an important role and are also known as indicator species to WNV infections, so their examination and research on the infections are essential.

During this Ph.D. work, we examined many aspects of the Hungarian West Nile virus situation in equines, and this dissertation summarizes our results. During the research, our main aim was to comprehensively overview the equine infections in Hungary, including epidemiology, diagnostics, treatment, and prevention.

According to the main topics, our work carried out four different studies. The first study, which is the most important and longitudinal, summarizes the epidemiology and clinical manifestation of WNV infections in horses. The second study describes the laboratory findings of the cerebrospinal fluid in infected equines, compared to healthy, control horses. In the third study, we summarized the treatment options, which were performed at stable circumstances. Finally, the fourth study includes recommendations on the vaccination protocols used for prevention in endemic areas.

The data collection started many years ago, but the last major outbreaks in 2016 and 2018 drew attention to the importance of a comprehensive study of the Hungarian situation. Our country plays a sentinel role in the European WNV circulation. Lineage 2, which now dominates most of the outbreaks in Europe and newly infected and endemic areas, is known to spread from Hungary. This fact and the amount of collected data help others at new endemic areas in the war against this virus and other flavivirus pathogens.

We hope this Ph.D. work and all the publications on the results may have a significant and valuable role in international literature.

Literature Review

West Nile virus (WNV) is a globally emerging pathogen belonging to the genus *Flavivirus* in the family *Flaviviridae*. The arthropod-borne single strained RNA virus is a member of the Japan encephalitis virus complex (JEV) and has a solid genetic relationship with other high-risk human and animal pathogens, like tick-borne encephalitis virus (TBEV) or Usutu virus (USUV) (Bakonyi et al., 2005, 2013). WNV and TBEV are endemic flaviviruses that regularly cause human, equine, and avian diseases in Hungary. However, USUV appearance has also been mentioned in the region (Bakonyi et al., 2005; Heus et al., 2020). Among the many flaviviruses, which can cause disease in mammals, the WNV has the most impact on equid health.

West Nile virus

Geographic distribution

WNV was first detected in the West Nile province of Uganda in the mid-1930s, and the name also comes from the location of the first detection (Smithburn et al., 1940). The presence of the WNV in Europe was first reported in Albania in 1958 after a specific antibody was detected in the blood of two local citizens. The first actual isolation of the virus took place in 1963 in the Rhône river delta (Hubálek & Halouzka, 1999). In later years, until the 1970s, the pathogen was also detected in wild birds, mosquitoes, humans, and horses and reemerged in the late 1990s (Chancey et al., 2015; Zeller & Schuffenecker, 2004).

According to phylogenetic analysis, eight divergent WNV lineages have been described (Pérez-Ramírez et al., 2017). WNV lineages 1 and 2 are the most widespread and have caused most of the major epidemics encountered so far (Beck et al., 2013). Identifying strains classified as lineage 3-8 WNV can be associated with vector surveillance activities (Kemenesi et al., 2018). The virus belonging to genetic lineage 1 is endemic in Africa, India, Australia, Europe, and North America. Within genetic lineage 1, strains 1a, 1b, and 1c are isolated. The virus belonging to genetic lineage 2 was previously isolated only in Africa, in areas south of the Sahara and on the island of Madagascar (Burt et al., 2002; Zeller & Schuffenecker, 2004; Botha et al., 2008). The first isolated WNV in 1937, also belonged to genetic lineage 2 (Lanciotti et al., 2002). Because of the occurrence of diseases caused by the virus belonging to genetic lineage 1 and the higher death rate, this genetic lineage was attributed to a greater ability to cause disease (Hernández-Triana et al., 2014). Based on experiments with mice, it was revealed that neuroinvasive phenotypes could be found in both genetic lineages. Between the

two lineages, 75% nucleic acid identity was discovered (Chaintoutis et al., 2019; Hernández-Triana et al., 2014).

WNV was a perfect example of how fast and unpredictable flaviviruses can emerge in a naïve population worldwide. WNV was introduced in New York City in 1999, causing dramatic outbreaks among humans, horses and birds. WNV has rapidly spread throughout the United States, causing more than 30,000 human and 24,000 equine cases only over a 10-year period (Chancey et al., 2015). Mass mortality of resident birds, especially crows, was also observed (Murray et al., 2010). Interestingly, in Europe, the epidemiological scenario in 1996–2010 was quite different from the one in North America, as epidemics were irregular and limited in time and space.

In Hungary, WNV caused the first outbreak in 2003 in a flock of geese and in 2004 in goshawks (Bakonyi et al., 2006). In Hungarian horses, WNV infection was first diagnosed in the autumn of 2007, and equine neuroinvasive cases have occurred every year since, with the number of cases varying widely. In the avian outbreak in 2003, the lineage 1 strain was identified with a solid genetic relationship to the strains isolated in 1998 in Israel and 1999 in North America (Calistri et al., 2010). Lineage 1 strains are responsible for the outbreaks mainly in the western hemisphere (Chancey et al., 2015), causing epidemics in France, Spain, and Italy between 2000 and 2010 (Hernández-Triana et al., 2014). An increase in WNV transmission and outbreaks, noticeable in Europe since 2010, has been associated with the spreading of WNV lineage 2 strains (Bakonyi et al., 2006). The first WNV lineage 2 strain was initially detected in Hungary in 2004. Since then all further Hungarian West Nile neuroinvasive disease (WNND) outbreaks have been caused by lineage 2 WNV strains, which lineage was previously only found in the Sub-Saharan region in Africa (Bakonyi et al., 2006). Genetically closely related lineage 2 strains caused human, avian and equine outbreaks in Greece (2010) (Bouzalas et al., 2016), Austria (2008, 2016) (Hernández-Triana et al., 2014; Heus et al., 2020), Italy (2011) (Hernández-Triana et al., 2014), France (2020) (Beck et al., 2020), Germany (2018) (Ziegler et al., 2019) and recently in the Netherlands (2020) (Vlaskamp et al., 2020).

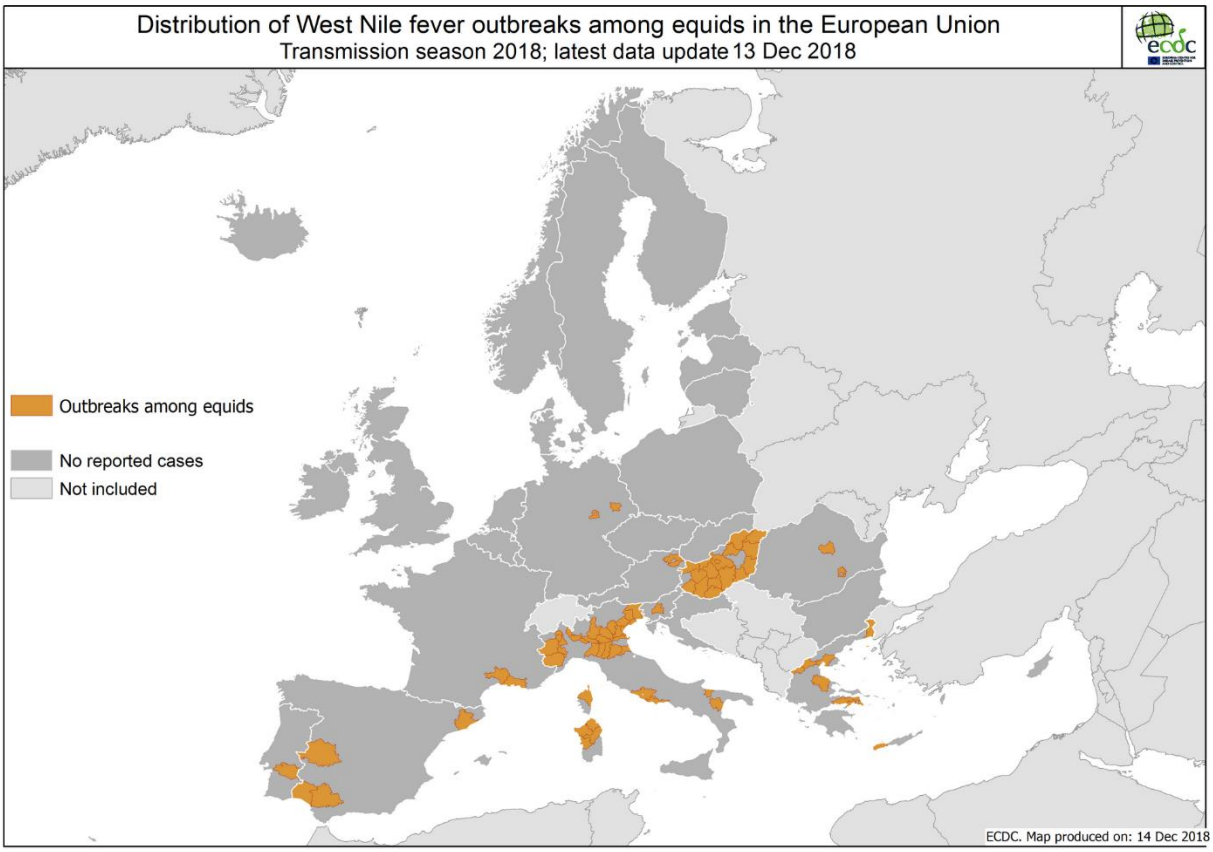


Figure 1. Distribution of West Nile virus outbreaks among equids in Europe, in 2018 (ECDC)

https://www.ecdc.europa.eu/en/publications-data/west-nile-fever-europe-2018-equine-cases-updated-13-december-2018

In Europe and in Hungary, the most significant transmission season was experienced in 2018, but previously in 2008 and 2016, human, equine and avian case numbers were increased. During the 2018 epidemic, 11 countries in the European Union reported confirmed cases among humans and horses (Figure 1.). In this last significant outbreak, previously not infected regions in Europe, like Germany and the Netherlands, became endemic, and some previously confirmed lineage 1 countries have reported lineage 2 infections. In Italy, WNV lineage 2 circulation was first documented in 2011 (Bagnarelli et al., 2011; Magurano et al., 2012). According to the high percentage of nucleotide homology (99.76%) between the Italian and French WNV strains, it was hypothesized that WNV lineage 2 gradually spread between 2017-2018 from Northern Italy to South-Eastern France (Beck et al., 2020) and then further west in the Mediterranean region, reaching Catalonia in north-eastern Spain (Aguilera-Sepúlveda et al., 2022). In Germany, this EU-dominant strain was first detected in 2018 in resident birds and horses (Bakonyi & Haussig, 2020; Michel et al., 2019). The first five locally acquired vector-borne human cases were reported in 2019 in the country. Another outbreak of nine locally acquired cases occurred in 2020 (Pietsch et al., 2020). WNV lineage 2 was reported in bird

and mosquito samples for the first time in the Netherlands at the end of August 2020 (Sikkema et al., 2020). Hereafter, the first locally acquired human WNV infections were diagnosed in the region of Utrecht (Vlaskamp et al., 2020). Regardless of the spread of dominant lineage 2 strains, lineage 1 strains may still be responsible for local outbreaks similar to the one in Spain in 2020 (Aguilera-Sepúlveda et al., 2022).

Transmission

Flaviviruses can be categorized into three groups according to their vectors: tick-borne, mosquito-borne, and viruses with unknown vectors. Among flaviviruses spread by mosquitoes, we can distinguish a so-called *Culex* clade and an *Aedes* clade based on the primary vector involved in transmission. The two groups also differ in terms of reservoir: in the *Culex* clade, birds are typically the primary amplifying hosts, and the viruses classified here are typically neurotropic pathogens that often cause meningoencephalitis in a vertebrate host (e.g., West Nile virus, Japanese encephalitis virus, etc.) (Beck et al., 2013). On the other hand, in the *Aedes* clade, non-human primates are the primary reservoirs, and most of the virus species classified here primarily cause non-neuroinvasive diseases (Beck et al., 2013). Overall, the spread of flaviviruses by both ticks and mosquitoes affects broad geographical areas, thus posing a significant burden on public and animal health. Among many flaviviruses which can cause disease in mammals, the WNV probably has the most impact on equid health (Cavalleri et al., 2022).

WNV is primarily transmitted by mosquitos belonging to the *Culex* genus, and more than 65 mosquito species can transmit the virus. These vectors are considered ornithophily species, as they mostly bite birds, but host switching to domestic animals also occurs. These mosquitos are also called bridge vectors because transmission between different species is possible. Adult mosquitoes carry the virus in their salivary glands, which are inoculated into birds or other vertebrate hosts during feeding. In this way, the inoculation of the pathogen can be continuous. The viremia that develops in susceptible vertebrates over a period of days provides an opportunity for feeding mosquitos to pick up the virus. Humans and horses are considered dead-end hosts of the virus, as in these species viremia phase is low and short, and mosquitos rarely become infected. There is a wide range of species of birds in which high levels of viremia can develop, and these may act as transmission species (Hubálek & Halouzka, 1999). The individual bird species show differences in terms of viremia and disease development. However, serological tests only provide an opportunity to determine individual bird species' sensitivity, so the infection's source cannot be determined.

Limited horizontal transmission via blood transfusion or organ transplantation is also documented in humans, and vertical transmission through the placenta and breast milk may also occur (Gould & Fikrig, 2004). These types of transmission have not been documented in horses until now.

Blood transfusion and organ transplantation can be a real threat, and testing of donors for possible flavivirus infections should have been part of the screening procedure. The first virus transmission via blood transfusion was detected in the United States in 2002. In this case, several recipients became infected with WNV. However, the donor was serologically negative for IgM, and only the virus could be detected from the blood samples by PCR tests (Pealer et al., 2003). Shortly after the first documented transmission in the United States, the PCR monitoring of blood donors was started for the presence of WNV (Pealer et al., 2003). In Hungary, the National Public Health Center (NNK) makes a considerable effort to work out screening methods for blood samples to determine flavivirus infections (Nagy et al., 2019). Blood transfusion and organ transplantation are all used in cases of immune-depressed recipients, in whom a low level of viremia can easily lead to a fatal outcome. Therefore, the screening procedures mentioned above have a massive role.

The virus begins to multiply in the salivary glands of mosquitoes. The process depends on the temperature and humidity, and in warm weather, it takes approximately 2 weeks. Trans-ovarian transmission of WNV has been identified in *Culex* and *Aedes* species as well, but this mode of transmission is probably not as important, although it also provides a source of WNV persistence (Castillo-Olivares & Wood, 2004). Low viral load has been measured in *Culex* mosquito larvae from the surrounding environment of infected horses (Zana et al., 2020).

In accordance with the activity of the arthropod vectors and the replication of the pathogen, seasonality can also be observed in the transmission pattern of WNV. Most clinical cases appear between August and November worldwide, depending on the presence of the vectors and the climate of the country (Castillo-Olivares & Wood, 2004). In Europe, WNV infections occur during the summer and autumn season (July-November) when vector species are abundant. Overwintering through winter diapause and hibernation of infected mosquito females may also likely occur (Paz & Semenza, 2013). In Figure 2, we illustrate the possible modes of transmission and hosts of WNV. According to current studies, the house sparrow (*Passer domesticus*) maintains the epidemic course of WNV both in Europe and North America, as it occurs in large numbers in both urban and outdoor environments (Castillo-Olivares & Wood, 2004).

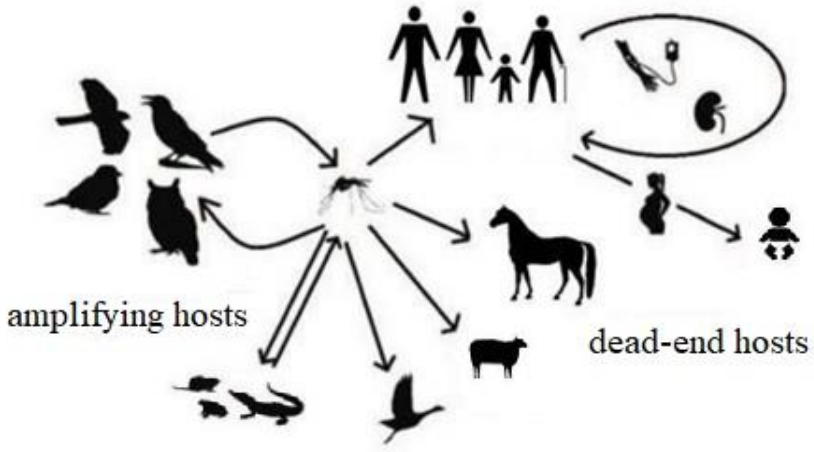


Figure 2. Transmission routes and hosts of West Nile virus

According to the present studies on hamsters, rats, and mice, the virus, inoculated into the skin with mosquito saliva, begins to replicate at the site of the bite, and then reaches the local lymph node and the lymphatic system with the help of Langerhans cells (dendritic cells). Once the pathogen enters the bloodstream, the primary viremia develops, and then the virus reaches other organs e.g. the spleen and kidneys. After WNV is present in more and more tissues and the virus titer in the serum has increased significantly, the pathogen reaches the central nervous system. After a week, the virus begins to clear from the serum and other organs, and in immunocompetent individuals, clinical signs are observed at that time (Castillo-Olivares & Wood, 2004; Kramer et al., 2007).

Clinical disease

Most of the human and equine WNV infections remain clinically silent. Approximately 1% of people infected develop flu-like symptoms or self-limiting febrile illness, and fewer cases end in the neurological form. Horses are considered to be more sensitive to WNV infection, but it is estimated that, 80% of the seropositive horses remain asymptomatic. Of those who develop illness, only approximately 10% develop severe neurological signs (Petersen & Roehrig, 2001; R. S. Porter et al., 2011; Sejvar, 2014). Although several descriptions of lineage 1 outbreaks have been published, less clinical reports on equine WNND caused by lineage 2 strains are available in the international literature (Kutasi et al., 2011; Murgue et al., 2001; M. B. Porter et al., 2003; R. S. Porter et al., 2011; Salazar et al., 2004; Snook et al., 2001; Venter et al., 2009; Ward et al., 2006).

The risk of developing severe illness has many factors, involving viral, host and also environmental circumstances. According to previous descriptions, there is no age, breed or gender predisposition among horses. In humans, it is known, that most of the severe neurological symptoms developed in the elderly. In humans, a much more detailed classification of WNV-caused illnesses is available (Sejvar, 2014). The infection can cause a flu-like syndrome called West Nile fever (WNF), which can be clearly differentiated from the neurological forms of meningitis (WNM), encephalitis (WNE), or poliomyelitis (WNP). A recent publication mentions age predisposition in different types of neurological forms. Data suggest that WNM involves younger age groups, while WNE is more typically manifested in older people (Sejvar, 2014). In the case of horses, identification of specific neurological forms is difficult because of the limited diagnostic modalities applied in these cases. In naturally infected horses, there appears to be no difference in the risk of clinical manifestations due to either lineage 1 or 2 infections (Cavalleri et al., 2022). Similarly, mortality rates in horses infected with WNV lineage 2 correlate with those reported for lineage 1 in Europe and North America (Kutasi et al., 2011; Venter et al., 2009; Ward et al., 2006). Based on a previous study in a rodent model, differences in pathogenicity between WNV strains are not related to phylogenetic lineage, geographic origin, or year of isolation (Pérez-Ramírez et al., 2017). Instead, virulence appears to be an evolving phenotype acquired independently of genetic background during virus adaptation to changing ecological niches (Cavalleri et al., 2022). Vaccines are protective against intrathecal or intradermal experimental infections, as vaccinated horses produced a lower level of viremia and did not have any clinical signs compared to control, unvaccinated ones. Outbreak descriptions also mention lower morbidity and mortality rates in vaccinated horses (Salazar et al., 2004).

The clinical manifestation of equine WNV infections can be highly variable, also in duration, severity, or combination of clinical signs. Based on previous descriptions of outbreaks in Europe and North America, WNND in horses is mainly accompanied by clinical signs such as fever, depression, ataxia, paresis, tremors, or recumbence. Ataxia is far the most frequent clinical sign, combined with weakness or muscle fasciculation. Ataxia may also appear in a symmetrical or asymmetrical way, such as when incoordination affecting fore- or hind limbs or all four. Obtund mentation, anorexia, low-grade fever or lameness are mostly the first general signs of WNND. Many publications mention colic in the initial phase of the disease, which can have a misleading effect on the diagnosis (Heus et al., 2020). Cranial nerve deficits such as facial paralysis, blindness, photophobia, and dysphagia are less observed signs (Figure 3.). Patients can get aggressive and have a change in their mentation associated with WNV infections. As residual signs, mostly lameness or mild grade ataxia appears.

In humans, severe headache, fever, fatigue, gastrointestinal complaints and all types of neurological symptoms can appear in WNV infections. Some types of WN syndromes can be associated with a self-limiting, maculopapular, non-pruritic rash over the torso and extremities. The exact reason of these rashes are not known yet, but it may correlate with host immune or cytokine response to infection (Sejvar, 2014). Due to differences between species, similar rash signs have not yet been observed in horses.

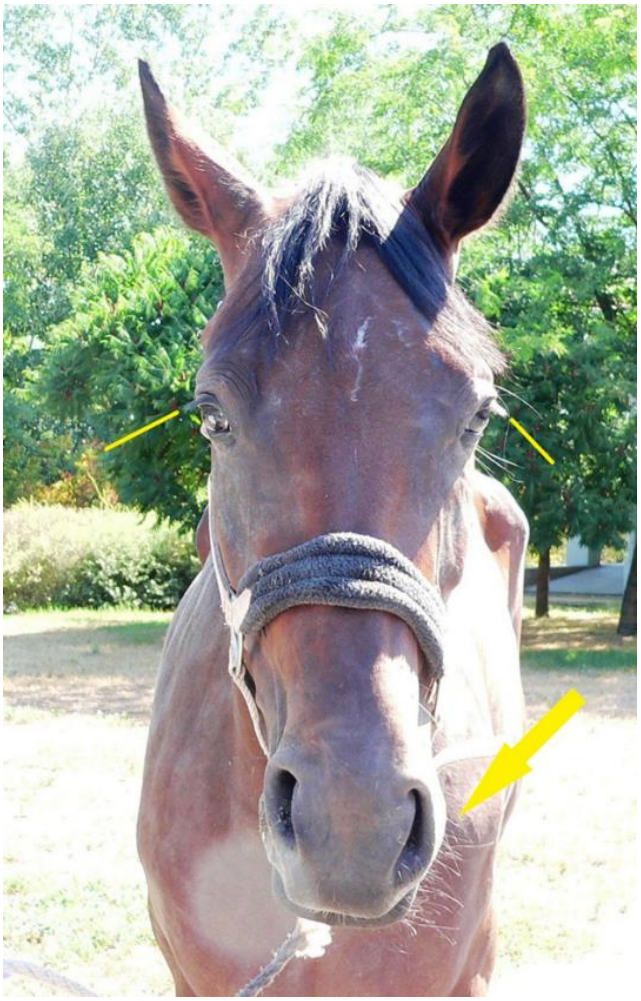


Figure 3. Left-sided, peripheral facial nerve paralysis in a horse, in Hungary

Diagnosis

Laboratory tests are essential for the diagnosis of the WNV infections because the clinical signs or any pathological findings are not characteristic of the virus. WNV infections and cases in any species require official notification of the Hungarian National Food Chain Safety Office (NÉBIH). Every horse with acute neurological signs during the mosquito season must be tested for WNV infection at officially accredited laboratories. In Hungary, only NÉBIH, as a national

office, has accredited laboratories, which are continuously reevaluated by a foreign commission from the World Organisation for Animal Health (WOAH, founded as OIE) (Beck et al., 2013).

Many different serological tools are available to diagnose or screen for WNV antibodies. Neutralizing antibodies have a critical role in the long-term protection from disease, and their measurement is well used for any flavivirus, including West Nile virus clinical diagnostics and surveillance. Due to the amino acid similarity in the E-protein, cross-reactions and cross-neutralization is observed between genetically related flaviviruses. Although cross-reaction can appear during serology tests, it is still considered the most important diagnostic tool during ante-mortem diagnosis in horses and in other species.

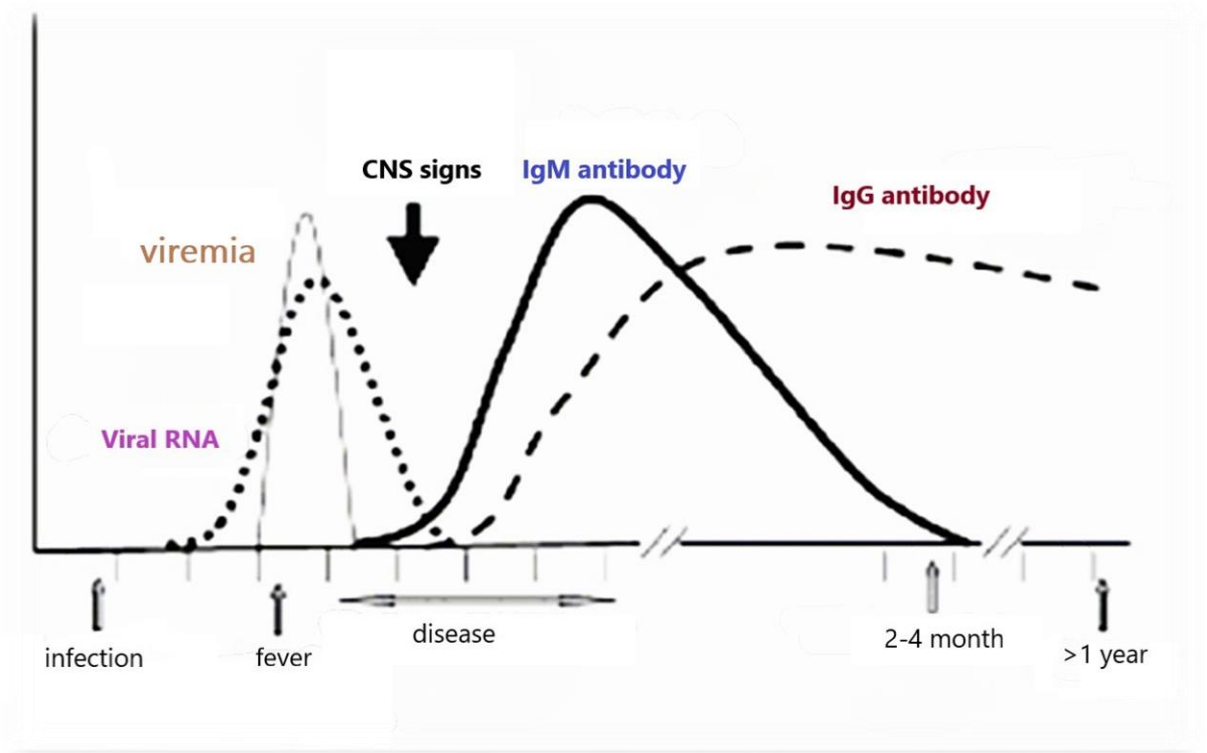


Figure 4. Immune response to West Nile virus infection

Enzyme-linked immunosorbent assays (ELISA) are preferred screening tools of flavivirus infections, because of their sensitivity, reproducibility, and rapidity. Ready to use commercial kits are available in veterinary and human medicine, for these inexpensive methods. Three different assays are in use, which are the competitive ELISA, the indirect ELISA and the IgM capture (MAC) ELISA. Figure 4. shows the presentation of different antibodies compared to the infection. IgM specific antibodies play an important role in the diagnosis of recent infections.

The concentration of IgM antibodies rises significantly few days after the infection and remains at a measurable level for approximately 4-6 weeks, but have been reported to occasionally persist for a year (Beck et al., 2013). Even after vaccination, the IgM antibody can be present in the blood for up to 52 days, so it is important to pay attention to the vaccination history in the case of serology tests (Joó et al., 2017). The IgM antibody can cross the blood-brain barrier, thus the serological examination of the CSF sample, which is for sure free of blood contamination, can lead to a truly reliable result. IgM antibodies can be hard to detect using VNTs and other less popular serological tools, and exhibit less cross-reactivity than IgG antibodies (Beck et al., 2013). The detection of anti-WNV IgM via MAC-ELISA is highly specific and sensitive, so the diagnosis of recent WNV infection often only requires a single test on serum and/or CSF. The detection of IgG antibodies with indirect ELISA requires the use of anti-species conjugated antibodies, while competitive ELISAs allow the testing of samples from any animal species. Competitive ELISAs are the most sensitive of all serological technologies developed, but they are best suited for screening purposes due to their lower specificity (Beck et al., 2013).

Plaque Reduction Neutralizing Test (PRNT) or micro Virus Neutralizing Tests (micro-VNT) are still the gold standard WNV serological tests. These laboratory techniques are highly specific, but are less sensitive than ELISAs. In this test, the ability of antibodies to reduce the number of lysis plaques in cell cultures, is visually quantified; a sample is considered seropositive if the threshold value (relative to the control) also achieved it. The disadvantages of these techniques are time consumption, and the need for Biosafety Level 3 (BSL-3) laboratories, which circumstances increase their cost as well (Beck et al., 2013). The evidence of IgM antibodies in serum or cerebrospinal fluid or the increase in IgG titers in two serial samples obtained 2–3 weeks apart is sufficient to confirm WNV infection in horses.

Other serological assays are less commonly used in everyday diagnostic or screening procedures, although these can also give reliable results. Hemagglutination Inhibition Test (HIT) is a fast, cheap and not BSL-3 required assay, which measure the ability of E-protein to aggregate erythrocytes in the absence of anti-E neutralizing antibodies. HITs are less sensitive, compared to ELISAs, and can have a high level of antibody cross-reactivity and cannot distinguish between IgG and IgM antibodies. Immunofluorescence Assays (IFA) are rapid, more specific assays, which may be used to differentiate between IgM and IgG antibody responses. Commercially available kits make its usage simple. Compared to ELISAs, IFA is more specific in detecting IgMs, but are on same level when detecting IgGs. Although there are recent publications on Microsphere Immunoassay (MIA), it is not used on a broad spectrum (Balasuriya et al., 2006).

Virus detection is a useful diagnostic procedure during necropsy, blood donor testing, or amplifying host surveillance programs. Horses and humans are considered dead-end hosts, and WNV detection from living animals is often more difficult than post-mortem diagnosis. This is because the viremic phase ended before the onset of clinical signs in many WNV infected animals, and the virus is rapidly cleared from blood following antibody production (Figure 4.). After death, WNV can be isolated or detected in brain material after being amplified using suckling mice or cell cultures and by detecting specific nucleotide sequences using reverse transcriptase-polymerase chain reaction (RT-PCR). Thalamus, hypothalamus, pons/medulla, and spinal cord contained West Nile virus in horses, but it has been detected with the highest mean RNA concentration in the medulla (Beck et al., 2013). Different PCR methods are well used in avian and mosquito surveillance and human blood donor screening programs. One of the important and novel experiences of virus detection tests in humans is that the virus can also appear in urine; it can be isolated from it and, compared to blood and cerebrospinal fluid samples, can be detected longer (Nagy et al., 2016). In horses, these new virus detection methods were unsuccessful, primarily because of the differences in urine physiology between species. Immunohistochemistry (IHC) and in situ hybridization are also appropriate techniques of WNV detection within formalin-fixed, paraffin-embedded brain tissue of horses; their use did not spread as wide as in the case of equine eastern encephalitis virus. Because of the low viral load in the brain tissue, it is advised to use several sections of the brain or the spinal cord. Collection of CSF and post-mortem dissection of infected horses can mean an increased danger to the examination staff, because the virus can be present in these examined tissues, so the use of biohazard equipment is obligatory to prevent infections (Beck et al., 2013).

In Hungary, the following diseases should be included in differential diagnostics: other flaviviruses (TBE, USUV, LIV) with the same seasonal appearance and clinical manifestation. Equine herpesvirus myeloencephalopathy (EHM) is also an endemic disease in Europe and in certain geographical regions rabies and Borna disease should also be considered. Alphaviruses, and equine protozoal myeloencephalitis have not been diagnosed so far in Europe. Bacterial meningitis, and botulism must always be taken into account. Non-infectious diseases should also be considered as differential diagnoses, because they can also lead to similar neurological signs as WNND. These are forage toxicities, selenium and vitamin E deficiency, wobbler's syndrome, and neurological symptoms resulting from degenerative or traumatic effects. Horses with wobbler’s syndrome or equine degenerative myeloencephalopathy have chronic progression and are more age-related than viral infections (Angenvoort et al., 2013; Castillo-Olivares & Wood, 2004).

Suspicion of WNV infection is usually considered based on the patient's provenance (a horse living in or originating from a WNV endemic area), the season, the patient's clinical presentation, and vaccination history (i.e., no or inadequate vaccination). Ancillary laboratory diagnostic tests are not specific in most cases, but these can be helpful in ruling out other neurologic or systemic causes. Mild, absolute lymphopenia in hematology usually appears in viral infections. Biochemical examinations are needed for the diagnosis of organ dysfunctions, and muscle-related enzymes may also be affected in cases of trauma or prolonged recumbency. CSF examination in horses (in contrast to human diagnostics) is not a frequently used procedure, and there are no specific abnormalities in the laboratory results (Cavalleri et al., 2022). Figure 5. describes the algorithm for diagnosing WNV infection in horses.

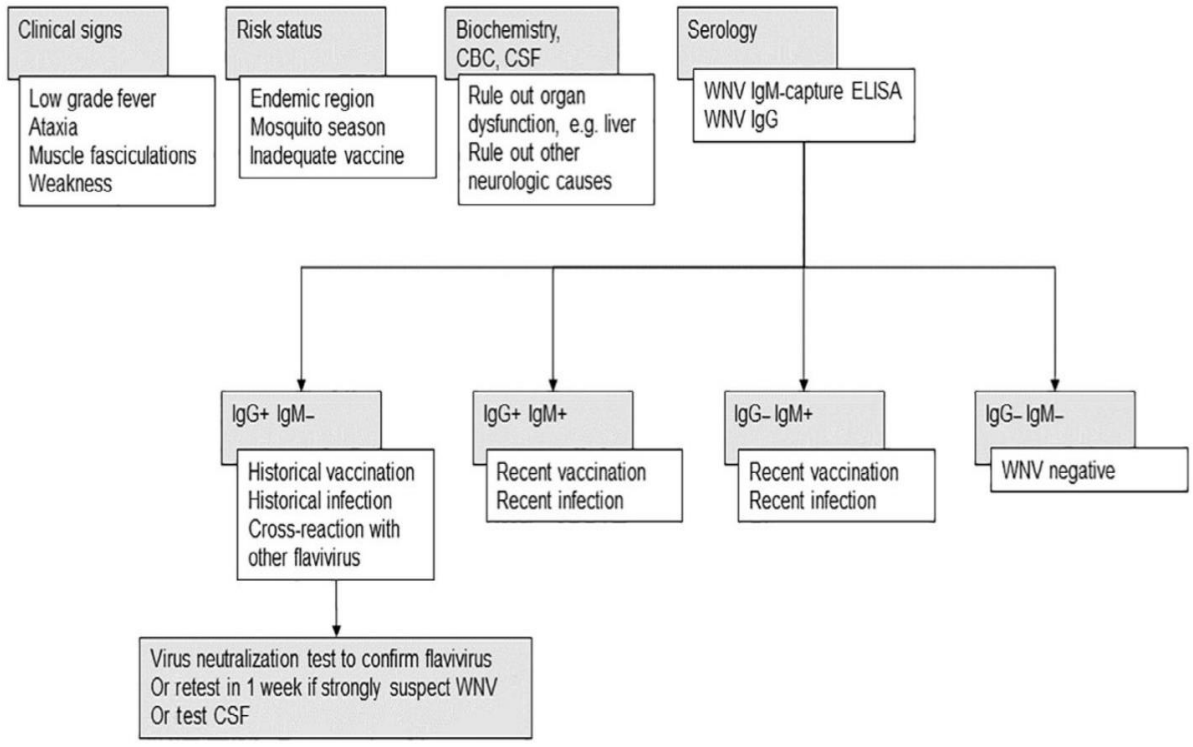


Figure 5. Diagnostic algorithm of West Nile neuroinvasive disease (Cavalleri et al., 2022).

Treatment

Treatment of West Nile virus infections in horses is mainly supportive and symptomatic because no specific treatment is available. The treatment includes the administration of antioxidant neuroprotective vitamin E, B, C, and selenium. Nonsteroidal anti-inflammatory drugs are often used, mainly at the introduction phase of the disease, to reduce inflammation

and relieve pain (flunixin-meglumine 1.1 mg/kg/12h/iv). There are divided opinions on the use of glucocorticoids in WNND because, on the one hand, their immunosuppressive effect might increase the level of viremia. However, on the other hand, the immunomodulatory effects benefit the reduction of immune-mediated inflammation of the nervous system. The glucocorticoid effect on viremia was confirmed in experiments in dogs (Bowen et al., 2006). Until today there is no scientific support or prohibition on using glucocorticoids. Dexamethasone sodium (0.05-1.0 mg/kg/24h/iv), as a short-acting corticosteroid, is often administered in case of recumbency or sudden worsening of the condition (Cavalleri et al., 2022). As part of complementary therapy, fluid and electrolyte administration can also be beneficial (R. S. Porter et al., 2011). The use of antibiotics can also be helpful in preventing secondary bacterial infections; however, care must be taken to reduce the use of antibiotics in order to prevent emerging resistance (R. S. Porter et al., 2011). There are experimental findings about antiviral agents and immunoglobulin therapy, mostly in humans, but their usage did not spread in veterinary medicine.

Prevention

In the case of vector-borne pathogens, one of the most essential and economical ways of protection is decreasing the vector-host interaction. Horses can be protected against mosquito spread WNV by stabling equines mostly during the evening, when the vectors are more active and use repellent, insecticide chemicals to decrease the mosquito pools. During entomological observations of infected horse cases, abundant larval breeding sites in the immediate surrounding of the horses (swimming pool with a neglected water body and precipitation pools in artificial containers) were discovered, and *Culex pipiens* larvae in the swimming pool were PCR-positive for WNV RNA (Zana et al., 2020). It is recommended to drain all shallow, stagnant water pools, like swimming pools, to decrease the number of vectors. These simple efforts can significantly reduce the number of infections in both horses and humans (Szentpáli-Gavallér et al., 2014; Zana et al., 2020).

Vaccination

In order to alleviate the growing threat of the virus, different types of vaccines are available to prevent the neurological manifestation of the disease in horses (Chaintoutis et al., 2015; Pearce et al., 2013). These vaccines decrease viremia and reduce the neurological signs in horses infected with WNV. In Europe, three different types of vaccines are available.

The first vaccine licensed in Europe (EMA/510730/2008) is an inactivated lineage 1 WNV vaccine (Zoetis Equip® WNV previously called Duvaxyn®WNV, Pfizer in Europe and West Nile-Innovator®, Zoetis in the USA), which reduces the number of viremic horses after infection with WNV lineage 1 and 2 and reduces the severity of clinical signs against WNV of lineage 2. Equip® WNV contains the lineage 1 strain New York 1999/VM2 and could effectively protect mice in a challenge with a neuroinvasive lineage 2 WNV strain isolated in South Africa (Chaintoutis et al., 2015; Venter et al., 2009). In other pilot studies Equip® WNV was capable to induce cross-protection in natural and experimental infections with virulent lineage 2 WNV strains in horses (Bowen et al., 2014; Chaintoutis et al., 2015). The second vaccine, which is available in Europe and in Hungary as well, is the Proteq West Nile® (EMEA/V/C/002005; Boehringer Ingelheim) which vaccine is a modified-live attenuated Canarypox recombinant vaccine, containing virus strain vCP2017 and expressing the WNV prM/E proteins. The third vaccine in use is the WNV Equilis®Prequenza containing inactivated chimeric Yellow Fever Flavivirus (YF-WNV) expressing structural prM/E proteins of WNV (EMEA/V/C/002241; Intervet International BV).

Vaccination is recommended from the age of 5-6 months, and all the vaccines mentioned above can be used in pregnant mares. In the case of prepartum immunization, the first vaccine should be administered before the breeding season and the booster a few weeks before foaling. Through maternal immunization, foals seem to be protected from the WNV clinical disease. Continuous booster vaccinations are needed to maintain protective immunity (Cavalleri et al., 2022; Chaintoutis et al., 2015).

One Health

Equines are considered approximately 10 times more sensitive to symptomatic WNV infections than humans. This fact raises the observation that equids may play an indicator, sentinel role in surveillance programs and are highly pertinent from a public health perspective. Therefore, passive surveillance strategies based on horses are suitable for predicting human health risks e.g., the screening areas of blood donors. Since previous studies pointed out Hungary as an important ecological niche for virus diversification and dissemination in our geographic area, there is an urgent need for a country-wide, organized surveillance system regarding West Nile virus (Chaintoutis et al., 2019; Zana et al., 2020).

Objectives of the thesis

Study I

The first retrospective case series study aimed to obtain an overview of the prevalence, seasonal and geographical distribution, and clinical manifestation of WNV infection in horses in Hungary.

Study II

The aim of the second study was to compare the biochemical and cytological findings of CSF analysis in horses with acute neuroinvasive WNV infections with those of the CSF from control healthy horses.

Study III

In the third study, we summarize the clinical diagnostic and treatment features of WNV neurological disease specific to Hungary and describe two cases of WNV neurologic disease with a particular focus on how recumbent neurological cases could be managed in stable conditions.

Study IV

The objective of the fourth study was to characterize the serum neutralizing (SN) antibody titers against a lineage 2 neuroinvasive WNV strain in response to vaccination with an inactivated lineage 1 vaccine in a European endemic area.

Study I

Clinical manifestation and epidemiology of West Nile virus infection in horses

Materials and Methods

This retrospective case series study and outbreak description is based on observation and data collection in the period of 2007-2020.

We used the following diagnostic criteria based on the World Organisation for Animal Health (WOAH, founded as OIE), Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2013, Chapter 2.1.20. West Nile Virus (OIE Manual). We considered laboratory-confirmed recent West Nile virus infection where WNV-specific IgM was detected in serum. Based on previous studies, IgM capture ELISA used in our studies, is more specific than other serological tests so misdiagnosis secondary to cross-reactions should be negligible (Beck et al., 2020; Pérez-Ramírez et al., 2020).

Clinical case of equine WNND was diagnosed, if acute neurological signs appeared during mosquito activity season and the horse tested positive for WNV-specific IgM in an officially certified laboratory. Sick horses were confirmed as clinical WNND cases if they met both criteria. Regarding laboratory cases, the only condition was the presence of the IgM antibody, but these horses were clinically asymptomatic.

Data and sample collection:

For geographical and seasonal analysis we used the database of the Hungarian National Food Chain Safety Office (NÉBIH) about all IgM positive horses. For evaluation of WNND characteristics, we used symptomatic horses, which were diagnosed according to the WOAH guidelines as described above. Attending veterinarians were contacted to fill out a standard examination questionnaire (see supplement material) about the confirmed WNND equine patients.

The general data (age, breed, sex, usage) of the patient, the vaccination history, the onset and type of general and neurological signs, sampling date, geographic origin, treatment and outcome were recorded. The exact date of laboratory sampling was considered as the onset of the disease, as all blood samples were collected in less than five days after the appearance of the first clinical signs.

Native blood samples were collected from the jugular veins of the horses and tested for the presence of IgM antibodies with a commercial Enzyme-linked Immunosorbent Assay (ELISA) (INgezim® West Nile IgM, EUROFINS INGENASA, S.A, Spain) (Beck et al., 2020; Pérez-Ramírez et al., 2020).

Equine veterinary practitioners carried out sample collection as part of their routine diagnostic work-up in seasonal neurological cases. Samples were also submitted from horses without any clinical signs if the owner asked for testing stablemates as well.

PCR analysis and definition of lineage:

Central nervous system (CNS) tissues of horses which were euthanized in clinical settings, were examined by nested reverse transcriptase (RT)-PCR or real-time RT-PCR to identify the viral pathogen as described by Kutasi et al. (Kutasi et al., 2011).

Data analysis

Two databases were used for our analysis. The first one contains the primary data (date of sampling, location, contact veterinarian) of all the ELISA IgM-positive horses from 2007-2020 in Hungary. The second database was filled with general clinical data and clinical signs of the available horses from the first dataset.

To assess temporal and spatial seropositivity patterns we used annual descriptive statistics on county level. We opted to not conduct hypothesis testing as data collection was not random and potentially biased. Nonetheless, we present descriptive statistics as the information and the patterns are of value. The relationship between clinical signs and survival was described using Classification and Regression Trees (CART) (Breiman et al., 2017; De'ath & Fabricius, 2000). Briefly, these methods rely on a recursive algorithm to partition data based on the explanatory variables, selecting the best splitting variable at each node. Here we fitted a classification tree on the survival status of the individual with all clinical sign presence/absence data as explanatory variables.

Results

Temporal and geographical descriptions of outbreaks were based on the data of 198 West Nile virus IgM-positive horses. Not all of the positive tested horses showed clinical signs during testing, as some horses had subclinical infection. Data of these horses were used for the evaluation of geographical and temporary distribution. Out of the 198 IgM positive animals, we were able to collect the complete clinical history of 124 equids with seasonal acute neurological signs by contacting their treating veterinarians.

In case of 6 euthanized horses, PCR screenings were successfully carried out. In all of the samples, the lineage 2 strain was identified. We included one horse in our study, which had an incomplete vaccination with only one dose of vaccine before the onset of clinical signs. No other horse had WNV vaccination history prior to infection.

Table 1. contains the exact numbers of the detailed equine WNND cases during our study period. The three highest case numbers in our study were collected in 2008 (16), 2016 (19), and 2018 (72).

Table 1. Number of clinically examined animals by years and distribution in the whole dataset

Year	**2007**	**2008**	**2010**	**2012**	**2013**	**2016**	**2017**	**2018**	**2019**	**Total**
Examined number of horses (n)	2	16	2	1	4	19	2	72	6	124
Percentage of cases according to years (%)	1.6	12.9	1.6	0.8	3.2	15.3	1.6	58.1	4.8	100

Geographical distribution

The geographical distribution overview was divided into two equal, seven years examination periods, 2007-2013 and 2014-2020, because of better illustration and the distribution of case numbers. In the first period, only a few counties reported equine cases of WNND. The origin of the first detected equine case in 2007, was the region of Hortobágy, which part of the country is well known of its migrating avian population (Trájer et al., 2014; Végvári, 2015).

The region of Hortobágy and county of Hajdú-Bihar stayed severely infected at the later period as well. The virus affected mostly the whole country in the second examination period, case numbers increased significantly in many areas of Hungary. Results can be seen in Figure 6.

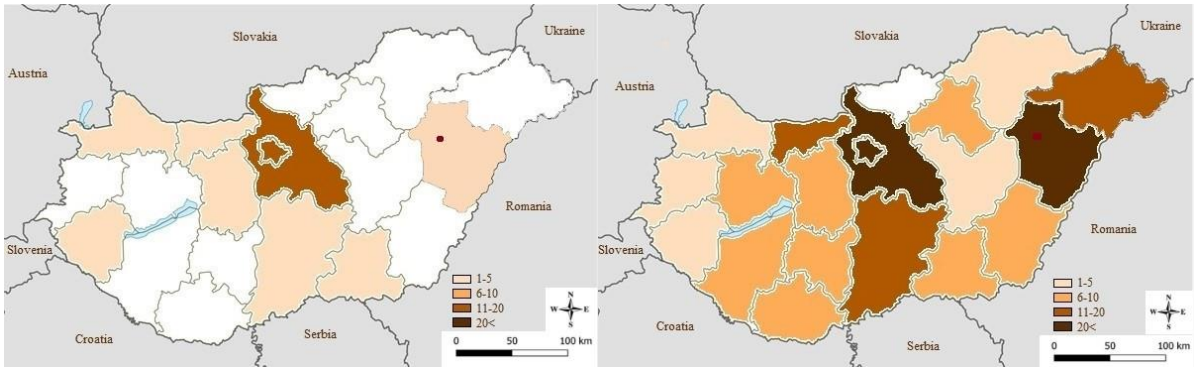


Figure 6. Geographical distribution in examination period 2007-2013 and 2014-2020. Origin of the first detected equine case in 2007 is indicated by a red mark (Hortobágy, Hajdú-Bihar County).

Temporal distribution

When examining the seasonal occurrence in the years with the highest number of cases (2008, 2016, 2018), no major change in seasonality was detected, but a trend could be seen that first cases occurred earlier in the season. During the first WNND outbreak in 2008, the first reported case was in the 35th week, in 2016 the first horse that has been tested positive was reported in the 31st week and in 2018 in the 26th week. The mean value of the seasonal period in these three years was at the 35th (2018) and 38th (2008, 2016) week. In the whole dataset, the mean of the seasonal peak was at the 35th week (day 245). Table 2. contains the collective results of seasonality.

Table 2. Collective seasonal data on the whole dataset

Year	**2007**	**2008**	**2009**	**2010**	**2012**	**2013**	**2016**	**2017**	**2018**	**2019**	**2020**
n	2	18	1	2	1	4	47	3	109	10	1
Mean (week)	44	38	33	30	40	36	38	35	35	32	38
Median (day)	306.5	264.5	229	213	275	244	267	253	242	225	261
First case (day)	306	239	229	213	275	213	215	219	178	214	261
Last case (day)	307	284	229	213	275	289	330	253	292	271	261

In Figure 7. the curves of each season can be seen. According to the sensitivity, the scale is divided by days. Only those examined years are displayed, where information on more than five cases where available.

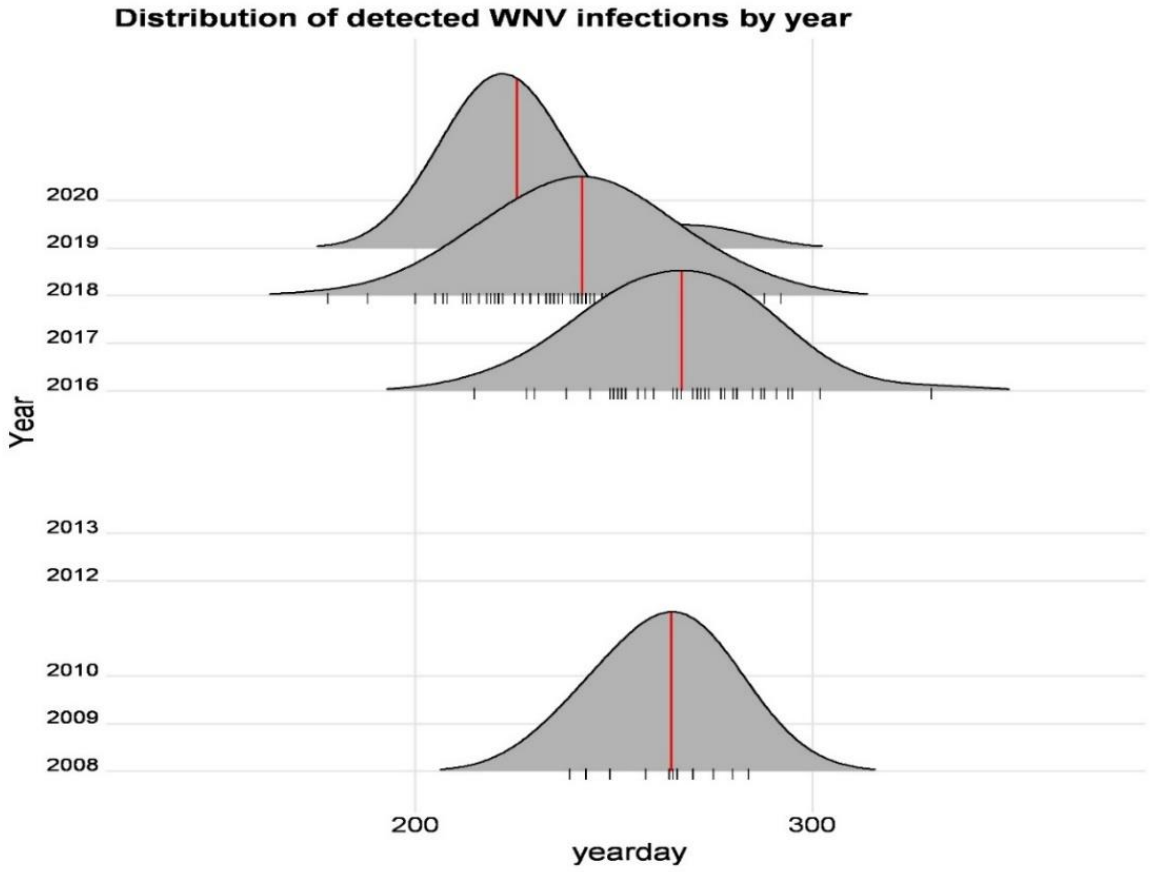


Figure 7. Distribution of detected WNV infections by year.
Vertical lines along the x axis depict individual observations, the curve is a result of less smoothing to aid the visualization of the sample distributions, while annual median year days are marked with red lines on the diagram.

Clinical data

The age of the horses with acute confirmed WNND ranged from 3 months to 22 years, with a mean age of 8 years. The age of the horses was sorted into three age categories, 32.3% were under 5 years of age; 55.6% were between 6-15 years, and 11.3% were above 15 years old. Sixty-seven (54%) were male, while 49 (39.5%) were female.

Apparent breed predisposition was not seen, 7 different breeds were represented in the database. Most horses were Hungarian warmbloods (n=84; 67.7%) and ponies (n=17; 13.7%), but many other breeds like Friesians (n=8; 6.4%), Arabians (n=6;4.8%), Thoroughbreds (n=4; 3.2%), draft horses (n=4; 3.2%), and Przewalski horses (n=1; 0.8%) were also affected.

The most common clinical sign was ataxia, which appeared in 81.5% (n=101) of the cases. Lameness of any leg or hindquarter paresis (12.1%) often preceded generalized ataxia. Both fore- and hind limb paresis and ataxia were recorded, but in many cases veterinarians could not distinguish between different types of ataxia. In the overall database hind limb ataxia was most frequent (n=40; 39.6%). General weakness was seen in many cases (62.1%; n=77) which manifested in some of the cases, as horses had shifted their weight continuously from one leg to another. Hyperesthesia appeared in 35.5% (n=44) of patients, and it was one of the most common and spectacular signs at the onset of the disease, and its severity ranged from very mild abnormality to sudden collapse on touching. Muscle fasciculation appeared to be a frequent clinical sign, in overall 37.9% (n=47) of the examined patients. Triceps muscle was the most affected region, but muscle fasciculation could affect the face, hind limb, and thoracic muscles or became generalized. Different clinical signs of cranial nerve deficits have been observed in our study. Twenty-five horses (20.2%) were affected by temporary facial nerve paresis/paralysis. Nystagmus and dysphagia were observed only in a minor proportion, in 3.2% and 7.3% of horses studied. In 43.5% (n=54) of the cases, behavioral changes were observed, including confused behavior, teeth grinding, and aggressive or self-harming behavior.

Most common initial clinical signs were lethargy and weakness, which appeared in about 62.1% (n=77) of cases. Hyperthermia (over 38.3 ℃ up to 40,0 ℃) was also a common sign, at the beginning of the disease. Horses, mostly along with fever, transiently showed loss of appetite, but generally, horses did not have anorexia after the initial phase, during the clinical progression. In 16.9% (n=21) of the cases, the first abnormality was the colic-like behavior, which appeared shortly before the onset of obvious neurological signs, like generalized weakness and ataxia. 35.5% (n=44) of the horses became recumbent at some point of the disease progression. Clinicians also reported hindquarter paresis with patients sitting in a dog position and were unable to stand up without specific aids. Even horses with prolonged recumbence could survive, if they were able to maintain sternal position and kept on eating during the critical phase of the disease. Thirty-one (70.5%) recumbent horses did not survive, of which comatose conditions appeared in 16.1% (n=5) of the cases. Horses with prolonged recumbency, collapse and comatose condition were euthanized on humane grounds in 61.9% of cases.

Table 3. contains the overall results and the annual distribution of each clinical sign. The signs are listed in descending order according to their total frequency of occurrence.

Table 3. Results of clinical signs.

The three years (2008, 2016, 2018) with the highest case numbers are displayed separately. The total number and percentage show the results found in the whole dataset.

Clinical sign	Total number (n)	Total percentage (%)	2008 (n; %)		2016 (n; %)		2018 (n; %)	
Ataxia	101	81.5%	11/15	73.3%	15/19	78.9%	60/70	83.3%
Lethargy/depression	77	62.1%	11/14	78.6%	10/19	52.6%	45/72	62.5%
Paresis /weakness	77	62.1%	10/11	90.9%	8/19	42.1%	47/72	65.3%
Behavior change	54	43.5%	9/12	75.0%	11/19	57.8%	20/72	27.8%
Anorexia	51	41.1%	6/14	42.9%	7/19	36.8%	29/72	40.3%
Hyperthermia	50	40.3%	3/15	20.0%	8/19	42.1%	31/72	43.1%
Muscle fasciculation	47	37.9%	8/12	66.7%	5/19	26.3%	30/72	41.7%
Recumbency	44	35.5%	7/12	58.3%	7/19	36.8%	27/72	37.5%
Hyperesthesia	44	35.5%	5/11	45.5%	6/19	31.6%	27/72	37.5%
N. facialis paralysis	25	20.2%	8/13	61.5%	3/19	15.7%	8/71	11.3%
Colic	21	16.9%	2/15	13.3%	6/19	31.6%	10/72	13.9%
Limb paralysis	16	12.9%	3/11	27.3%	2/19	10.5%	10/72	13.9%
Lameness	15	12.1%	0/16	0.0%	8/19	42.1%	4/72	5.6%
Dysphagia	9	7.3%	1/15	6.7%	1/19	5.3%	4/72	5.6%
Nystagmus	4	3.2%	0/14	0.0%	0/19	0.0%	2/72	2.8%

Information on outcome was available for 122 of the 124 horses included in the clinical study. Overall, 68.5% (n=85) of horses recovered and 29.8% (n=37) died during the acute phase of the disease. During the biggest outbreaks, 68.6% (11/16) in 2008, 68.4% (13/19) in 2016 and 69,4% (50/72) in 2018 survived. Difference of survival probability among age categories was not significant (X2=1.77; df=2; p-value=0.4121). The youngest 0-5 years old horses 62.5% (25/40), 74.6% (50/67) 5-15 years old, and 71.4% (10/14) of the 16< years old horses survived.

Foals (i.e., animals ≤ 1 year old) were more likely to die (66.7%; 6/9) as a consequence of WNV infection than older animals. The CART fitted on survival probability by clinical signs showed that if recumbence and anorexia were both present, all individuals deceased. In case of recumbency without anorexia, approximately 50% of the individuals survived while the lack of recumbency resulted in over survival probability of 90%. In Figure 8. CART result is displayed.

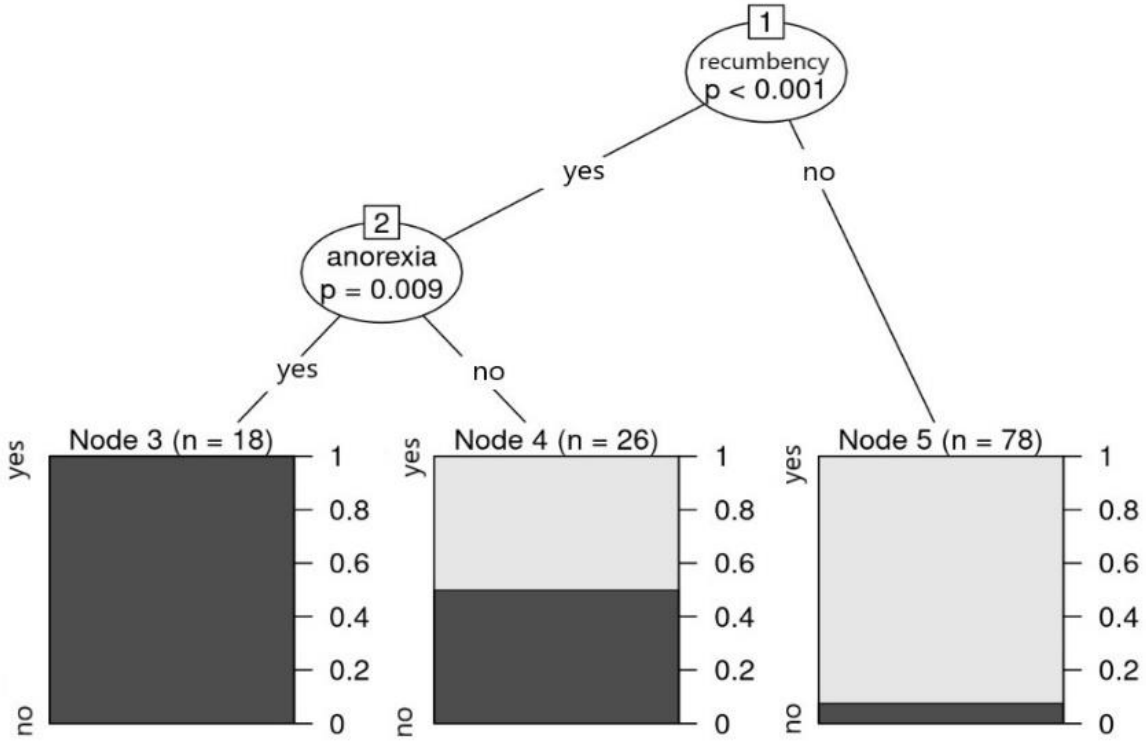


Figure 8. Decision tree on survival status according to all clinical signs. Recumbency and anorexia are two clinical signs that were able to categorize survival status. If both were present, the survival probability was 0 in our population.

Follow up of recovery was only available in the cases of 39 horses, due to the temporally long data collection period. In the examination period between 2007 and 2013, 26.7% (4/15) of the affected horses still had residual signs at least 2 years after the recovery of the acute phase of WNND. After the 2018 outbreak, 25% (6/24) of the survived equines had some residual sign related to previous WNND. These signs were mild hind quarter ataxia (3/6), weakness (1/6), and behavior changes (2/6).

Treatment

Treatment protocols were divided into categories according to the administration of non-steroid anti-inflammatory drugs (NSAID) or glucocorticoids. In Group 0 (12.5%) the animals did not receive any medication during the disease. Group 1, which included 46 horses (37.1%), were treated with NSAID drugs, while Group 2, with 48.4% of all patients, horses received glucocorticoid drugs at least once during the acute phase of the disease. We have analyzed the outcome of the disease in connection with treatment protocols. There was no significant correlation shown by the Pearson's Chi-squared test (X2=3.93; df=2; P-value=0.1397) between the treatment protocols and the survival rate of the WNND in horses. Results are displayed in Figure 9.

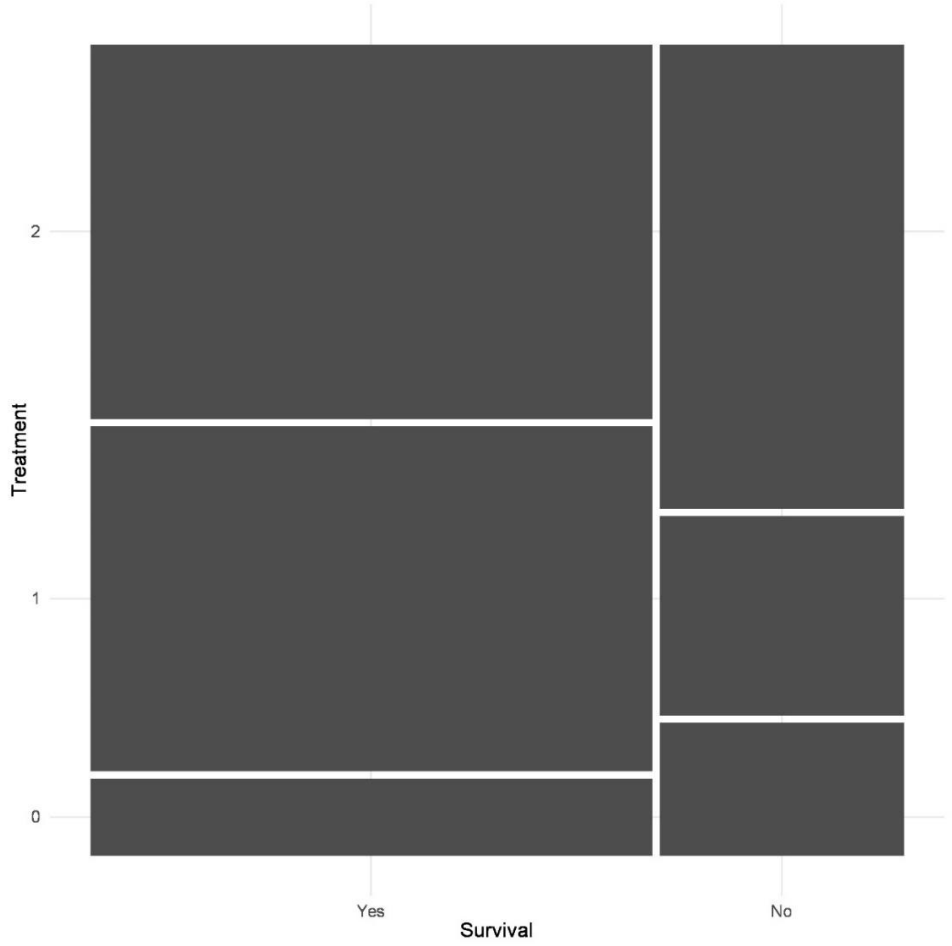


Figure 9. Mosaic plot of treatment protocols and the outcome of the disease (0=no treatment; 1=NSAID treatment, 2=glucocorticoid treatment). The area of the rectangles are proportional to the relative frequency of the given variable combinations

Discussion

We provide a comprehensive report on West Nile virus infections of equids in Hungary from the first detection through the massive transmission seasons in 2008, 2016, and 2018 until 2020. Our study includes temporal and geographical distribution of 198 horses, tested positive for WNV IgM and clinical description of 124 WNND cases with information on the general data and clinical signs of the affected equines.

The risk of a WNV-caused outbreak is multifactorial; the viral, host, and environmental circumstances all need to be taken into account. In our study, covering 13 transmission seasons, all the CNS samples tested for PCR, proved to be lineage 2 strains, during multi-approach research, trapped mosquitos adjacent to equine WNV cases proved to be infected with the same lineage 2 strains (Zana et al., 2020). These lineage 2 strains serve as origin of several epidemics across Europe (Italy, Austria, France, Germany, and the Netherlands) (Beck et al., 2020; Heus et al., 2020; Lecollinet et al., 2019; Sikkema et al., 2020; Ziegler et al., 2019). The epidemiology of these European lineage 2 strains is similar to the lineage 1 strains, those which were detected in North America and had spread all over the American continent. However, lineage 1 strains circulate in Europe as well, but they rather cause geographically and temporally localized outbreaks in Spain, Italy, and France and do not spread to more distant regions (Casimiro-Soriguer et al., 2021; Lecollinet et al., 2019; Murgue et al., 2001). Hungary reports human and equine WNND cases every year and the whole country became endemic during the past years. Both in humans and horses, the same pattern in incidence and geographic distribution can be seen (Nagy et al., 2016; Zana et al., 2020). Significant outbreaks during the 2016 and 2018 transmission seasons drew the attention to this pathogen and to the fact that the occurrence of new outbreaks is largely unpredictable. On the other hand, we might need to take population seropositivity into account. It raises the hypothesis that after a massive outbreak, individuals of different species might get asymptomatically seropositive and such limiting the spread of the virus. Looking at the geographical spread, newly infected areas may also occur in relation to vector and host density, and their interactions. The Hungarian capital and the surrounding areas were heavily affected during all outbreaks. Equine population densities can explain the background of this observation but it can be also explained by economical reasons. Horse owners around the capital have better access to veterinary care, information, and diagnostic techniques. Hajdú-Bihar County and the area of Hortobágy are well known for their migratory avian population, which carries the continuous risk of new virus introductions from Africa and where the birds' population density also favors virus transmission (Paz & Semenza, 2013). Overwintering of the mosquito species and temperature changes

strongly correlate with virus enrichment in an optimal area (Paz & Semenza, 2013). Diversity change has an important role not only in terms of territory but also in terms of seasonality. If many migratory birds leave in the autumn, viremia may increase in the remaining birds, and thus the density of affected and susceptible species and the infection of these secondary species (birds, humans, horses alike) may increase (Swaddle & Calos, 2008). The seasonal appearance of WNV infection has a strong parallel relationship with the vector activity and climate circumstances in the observed area (Paz & Semenza, 2013). Although the seasonal onset of WNV infections appears earlier than previously, the highest incidence rate is still between August and October. It is important to consider the current seasonal variations when developing vaccination strategies. Since the virus spreads from year to year to new European areas mainly in the northwest, it should be recommended to include WNV vaccines in vaccination protocols even in non-endemic areas of Europe. Especially when horses traveling to known endemic areas.

A publication on human WNND states that the risk of developing a severe neurological form of the disease is higher in the elderly (Sejvar, 2014). A similar pattern of the relative risk, according to age groups could not be demonstrated in our study. The mean age of horses was 8 years old, and other studies describing equine outbreaks publish similar findings with mean ages of 6.9 (Weese et al., 2003); 8 (Ward et al., 2006), and 9.5 years (Salazar et al., 2004). The reason why middle-aged horses are overrepresented in most of these studies is multifactorial. On one hand, it might reflect the age distribution of the horse population, on the other hand; foals, yearlings, and retired older horses are usually kept on pastures with less surveillance, so cases might be undetected. We had 9 affected foals under 12 months of age with a fatality rate of 66,7% and these results suggest that younger animals are less likely to survive. In some other studies a higher mortality rate was observed in the older population with a mean age of 10.8 ± 7.8 years (Salazar et al., 2004).

Hungarian warmblood horses appear to be somewhat overrepresented in the present study, and other breeds appeared to be underrepresented. Breed predisposition showed the composition of the Hungarian horse population rather than the predisposition to infection and due to small numbers of individual breeds, statistical associations could not be made. The same observation on each local breed predisposition has been mentioned in other studies as well (M. B. Porter et al., 2003; Salazar et al., 2004).

Previous studies in humans and horses have shown that males are more likely to develop the neurological forms than females. In the present study male equids were overrepresented also, but we could not demonstrate a significant association (Heus et al., 2020; Murgue et al., 2001; Ostlund et al., 2001; M. B. Porter et al., 2003; Salazar et al., 2004; Sejvar, 2014).

In human medicine, several manifestation categories have been described for WNV infections, such as WN Fever, WN Meningitis, WN Encephalitis and WN Poliomyelitis (Sejvar, 2014). The same classification cannot be used in horses, as the signs are more complex and the diagnostic possibilities are less sensitive for these kinds of differentiations. Fever, depression, and anorexia are considered general immune system activities and occur during or immediately after the viremia (M. B. Porter et al., 2003; Sejvar, 2014). Horses may only be febrile for a short period, likely early in the disease, and fever may be associated with viremia. These cases of West Nile Fever clinical presentation possibly go unnoticed, where short-term hyperthermia or depression is not detected and only horses with more spectacular signs receive veterinary care. West Nile Fever without any other neurological signs can occur in equines, based on the observation of a previous study, where WNV IgM positively tested horses had only fever (Bertram et al., 2020). In our study, we have only used the clinical data of horses with acute neurological signs and might have dismissed cases of West Nile Fever.

Clinical manifestation of the WNND in horses, reported in the present study, was similar to those reported in previous publications and ataxia was the most common clinical sign (Bouzalas et al., 2016; Heus et al., 2020; Kutasi et al., 2011; Murgue et al., 2001; Ostlund et al., 2001; M. B. Porter et al., 2003; Salazar et al., 2004; Snook et al., 2001; Venter et al., 2017; Ward et al., 2006; Weese et al., 2003). Although both lineages cause gait incoordination, our study and other publications on lineage 2 (Bouzalas et al., 2016; Heus et al., 2020; Venter et al., 2009) observed a higher, 100% and 82% presence of ataxia compared to studies describing lineage 1 outbreaks with an occurrence rate of 57% and 69% (Salazar et al., 2004; Ward et al., 2006). Both fore- and hind limb ataxia can be observed during outbreaks and the ataxia can be pronouncedly asymmetrical and can affect all four limbs as well (Kutasi et al., 2011). Primary forelimb ataxia is repeatedly described in lineage 2 outbreaks (Heus et al., 2020; Kutasi et al., 2011) but may also occur in lineage 1 cases (Salazar et al., 2004). Other movement disorders may appear in various ways, from mild lameness to severe paresis. Both weakness and paresis are characteristic neurological signs of the disease, affecting 62% of our patients, which observation was consistent with the findings of other publications (Salazar et al., 2004; Venter et al., 2009). Ataxia and weakness are reflection signs of brain and spinal cord disease. It may appear as part of direct infection of the spinal cord, interruption of motor tracts in the hindbrain, or loss of fine motor control with the infection of the large nuclei of the

thalamus and the basal ganglia (M. B. Porter et al., 2003). Weakness has been found to progress to recumbency in approximately one third of the cases. When no loss of consciousness occurred and the recumbent horses were forced to stand up with the help of slings or at least kept in sternal recumbency, the survival rate increased.

In one equine patient, a progressively worsening hindquarter paresis was observed 2 months after recovery from mild neurological WNV disease in the 2018 epidemic season. In humans, it is described that worsening of nervous system signs may appear in long term, which are presumably not caused by the presence of the virus, but rather have an immune-mediated origin. Paresis or paralysis can be part of the acute disease, but we hypothesize that these later signs are part of an immune-mediated reaction. The affected horse survived the disease and without histopathological examination, this hypothesis cannot be confirmed.

Hyperesthesia and muscle fasciculation were frequently detected signs and their incidence rate was above one third of the cases. The localization of these spectacular signs in our study was similar to what has been described by others (Heus et al., 2020; Salazar et al., 2004; Snook et al., 2001; Ward et al., 2006). In human cases, muscle fasciculation also has been mentioned as part of West Nile Encephalitis (Sejvar, 2014), and it can be one diagnostic criterion in horses with WNV encephalomyelitis. The pathogenesis of this abnormality likely includes loss of fine motor control (M. B. Porter et al., 2003).

Equine herpesvirus myeloencephalopathy (EHM), which is also a relatively common cause of neurological signs in horses in this geographic region, should be considered as a differential diagnosis. The clinical signs are well-differentiated in some cases, like the lumbosacral signs of EHM (hind limb ataxia, weakness, bladder paralysis) are very different from those induced by the brainstem damages of horses with WNV infection (four limb ataxia, muscle fasciculation, hyperesthesia), but there may be overlaps in the clinical picture in some cases, which is why background laboratory tests are essential for differentiation. Epidemiological features such as e.g. seasonality or co-morbidity of several horses in the same place may also be helpful to distinguish EHM from WNND.

Even though facial nerve paralysis, dysphagia, and nystagmus are considered common signs of WNND, they appeared scattered during our study. In the absence of histopathological examinations, we cannot declare for sure, the exact location of brain injuries, but according to previous publications, histological lesions within the pons and medulla oblongata can explain clinical deficits of cranial nerves. Other publications on lineage 2 WNND mentioned facial nerve paralysis twice as often, as we have experienced (Heus et al., 2020), although the appearance

of this sign differs in a wide range in other publications (Ostlund et al., 2001; M. B. Porter et al., 2003; Salazar et al., 2004). Dysphagia and nystagmus were uncommon and were reported with the same incidence as in other countries (M. B. Porter et al., 2003; Salazar et al., 2004).

Colic-like behavior was a common initial conspicuous sign of the disease. This sign often appeared first, persisted for a short time before the severe neurological disorders occurred, and sometimes had a misleading effect on the veterinary examination in field circumstances. Our reported gastrointestinal signs are consistent with previous observations of equine epidemics (Heus et al., 2020; M. B. Porter et al., 2003). Colic-like behavior can be a behavioral disorder that appears as a central nervous system sign, but also can appear as real colic with abdominal pain, which is caused by an injury to the autonomic nervous system. In hamster models, WNV was successfully isolated from the myenteric neurons. Increased contrast retention in the stomach compared to control hamsters supports the observation that gastrointestinal muscles may receive less nerve stimulation from the myenteric plexus (Wang et al., 2011). In addition to gastrointestinal dysfunction, damage to the autonomic nervous system is also supported by the observation that heart rate variability (HRV) diminished and cardiac arrhythmias have occurred in WNV-infected hamsters and humans (Sejvar, 2014; Wang et al., 2011). HRV is an appropriate clinical marker of autonomic dysfunction. Different types of behavior changes frequently illustrate the involvement of different parts of the brainstem (M. B. Porter et al., 2003; Sejvar, 2014). In addition to colic-like behavior, self-harming, aggressive behavior, and narcolepsy, deep depression were noticeable signs in our study. Others also mention these disorders during the acute phase of WNND or also as residual signs (Heus et al., 2020; M. B. Porter et al., 2003; Salazar et al., 2004).

Although we could not significantly detect it in the results, Figure 3 shows, that glucocorticoid-treated animals were less likely to survive the disease. Our observation is not consistent with the previous description of lineage 1 epizootic, where Porter et al. published opposite results (R. S. Porter et al., 2011). There may be several reasons for the difference, but it is likely that the relatively low number of cases in both studies impairs the success of an accurate comparison. The timing of glucocorticoid administration may also affect the results. In our study, horses were mostly given this type of medication when CNS signs were fast progressive leading to severe weakness or recumbency, and it could be assumed that administration of glucocorticoids earlier during the disease progression might increase its efficacy. On the other hand, our results might reflect a real negative effect on the survival and may even worsen the condition of patients by increasing viremia as it was shown in dogs (Bowen et al., 2006). We cannot claim that WNND has a specific treatment, but we observed that an infection detected

at an earlier stage and a well-organized supported therapy may have a beneficial effect on the outcome.

Collective survival rates differ on a wide range, mostly depending on the number of examined equines, rather than on lineages. Comparing our findings to studies with a large number of cases, the fatality rate stays relatively at the same level, around 25-30% (M. B. Porter et al., 2003; Salazar et al., 2004). We hypothesize, that descriptive publications on local outbreaks with a small number of cases rather show the most severe cases, with a higher rate of poor prognosis (Heus et al., 2020; Venter et al., 2009). On the other hand, almost all studies state that recumbency seems to be the best marker of fatal outcome, which observation concurs with our findings. Furthermore, we found that horses without the loss of appetite and with adequate support were able to be fed in a recumbent position, and had a higher chance for survival. In the background of this observation, we assume the more severe brainstem lesions lead to a fatal outcome. Another remarkable observation is that approximately 70% of the death are euthanized for humane reasons, which is also mentioned in most of the reports. The background of this high amount of euthanasia should be taken into account on a case-by-case basis, and usually it is a consequence of a poor prognosis with long, costly treatment.

Conclusion

West Nile virus has been an endemic pathogen in Hungary in the last 14 years, causing severe outbreaks among the equine and human population all over the country. According to the higher susceptibility, the examination of equines may play an important role in predicting outbreaks. An overall surveillance system in Hungary that includes both disease and PCR screening results would provide important information for both veterinary and human medicine. WNV infection is recognized on time, and a well-organized supported therapy is an essential factor of the successful outcome of the disease. Future studies may use equine cases as an indicator of WNV-intensive transmission activity and epidemiological and entomological studies to further understand the risk factors of WNV epidemic transmission. The collective results of this research can give a comprehensive overview of the Hungarian equine WNND cases and serve as the base point for collaborative inter-discipline research and programs on West Nile virus. Our work also reflects the urgent need for a national, organized surveillance system related to West Nile virus in the One Health approach.

Study II

Characterization of the cerebrospinal fluid of horses with West Nile virus neuroinvasive disease

Materials and Methods

The data were obtained during an observational retrospective case-control study between 2011 and 2016. Since WNND occurs seasonally in Hungary (Kutasi et al., 2011), data were collected between August and November each year. The 20 horses in the control group were sampled simultaneously in the same period. The Animal Health and Welfare Directorate of the National Food Chain Safety Office (22.1./1606/003/2009) permitted the study. WNV neuroinvasive cases were defined on the basis of seasonality (August–November), acute neurologic clinical signs (less than 5 days), positive serum IgM ELISA test (IDEXX IgM WNV Ab Test, Hoofddorp, The Netherlands), and the absence of any WNV vaccination in the history. Only clinically healthy horses without any neurological signs and with hematological and biochemical parameters within the reference range were included in the control group. In the control group we did not include privately owned horses: the control animals were owned by the university and kept for research purposes in the stables of the clinic and at the neighboring university teaching farm. Age, breed and sex characteristics of the horses and the sampling sites are described in Table 4.

Table 4. Age, breed, gender characteristics and sampling sites.

Group	Age	Breed	Sex	Sampling site
WNV-affected	M: 7.53 years	12 warmbloods	9 mares	8 lumbosacral
	SD: 2.84	1 pony	4 geldings	7 atlanto-occipital
Control	M: 8.94 years	16 warmbloods	10 mares	12 lumbosacral
	SD: 3.57	3 draught horses	10 geldings	8 atlanto-occipital
		1 thoroughbred		

M: mean; SD: standard deviation

Table 5. Clinical characteristics of West Nile neuroinvasive disease cases

No.	Outcome	Abnormal behavior	Cranial nerves affected	Hyperesthesia	Muscle fasciculation	Ataxia 1 = forelimb 2 = hindlimb 3 = all	Paresis 1 = forelimb 2 = hindlimb 3 = all	Hematology	Biochemical parameters elevated	CSF sampling site	VNT on CSF	PCR (CNS)
1	Euthanized	Yes	No	Yes	Yes	1 (3)	1 (3)	Leucocytosis, lyphopenia, neutrophil granulocytosis	TP, CK, glucose, lactate	AO	NA	Positive
2	Euthanized	Yes	Yes	Yes	Yes	1 (3)	1 (3)	Lymphopenia	glucose, CK, LDH	AO	Negative	Positive
3	Euthanized	Yes	Yes	Yes	Yes	1 (3)	1 (3)	Leucocytosis, lyphopenia, neutrophil granulocytosis	glucose, CK, LDH	AO	Negative	Positive
4	Euthanized	No	Yes	Yes	Yes	3	3	Lymphopenia	glucose	AO, LS	NA	Positive
5	Euthanized	No	No	Yes	Yes	3	3	Lymphopenia	TP, glucose	AO, LS	Negative	Positive
6	Euthanized	No	Yes	Yes	Yes	1 (3)	1 (3)	Lymphopenia	glucose, CK, LDH, creatinine	AO	Negative	Positive
7	Survived	No	Yes	Yes	Yes	2	2	Negative	LDH	LS	Positive	NA
8	Survived	No	Yes	Yes	No	2	2	Lypmhopenia	CK, LDH	LS	Positive	NA
9	Survived	No	Yes	Yes	No	3	2	Lymphopenia	TP, glucose, CK, LDH	LS	Positive	NA
10	Survived	Yes	Yes	Yes	No	3	2	Leucocytosis, lyphopenia, neutrophil granulocytosis	LDH	LS	Positive	NA
11	Survived	Yes	Yes	Yes	Yes	3	3	Lymphopenia	glucose, CK, LDH	AO	NA	NA
12	Survived	Yes	Yes	No	Yes	2	2	NA	NA	LS	NA	NA
13	Survived	No	Yes	Yes	Yes	2	2	Lymphopenia	Negative	LS	Positive	NA

Na: not available; TP: total protein; CK: creatine kinase; LDH: lactate dehydrogenase; CSF: cerebrospinal fluid; AO: atlanto-occipital puncture; LS: lumbosacral puncture; VNT: virus neutralization test, negative <1:2, positive >1:4; PCR (CNS): polymerase chain reaction on central nervous tissue specimens (all PCRs run on peripheral blood leucocytes were negative).

The CSF of all WNND cases was sampled within 36 hours of clinical admission, and the sampling site was determined on the basis of clinical signs (Table 5). The CSF of horses with characteristics of more pronounced brainstem and cerebral involvement was sampled by atlanto-occipital puncture under general anesthesia using 1 mg/kg xylazine iv (Xylasol, CP-Pharma GmbH, Burgdorf, Germany), 0.02 mg/kg butorphanol iv (Alvegesic, Alvetra u. Werfft GmbH, Vienna, Austria) and 1 mg/kg ketamine iv (CP-Ketamin, Produlab Pharma, Raamsdonksveer, The Netherlands) and that of horses with spinal cord involvement was sampled by lumbosacral puncture under sedation with 0.3–0.4 mg/kg xylazine iv and 0.02 mg/kg butorphanol iv simultaneously with lidocaine (Lidocain-human, TEVA, Debrecen, Hungary) local anesthesia as previously described (Schwarz and Piercy, 2006). In case of diffuse CNS involvement, both locations were used for sampling purposes, under general anesthesia. Altogether 15 samples were collected, and two horses underwent both lumbosacral and atlanto-occipital puncture. Within the control group, 8 animals underwent atlanto-occipital puncture under general anesthesia and 12 horses had lumbosacral puncture under sedation.

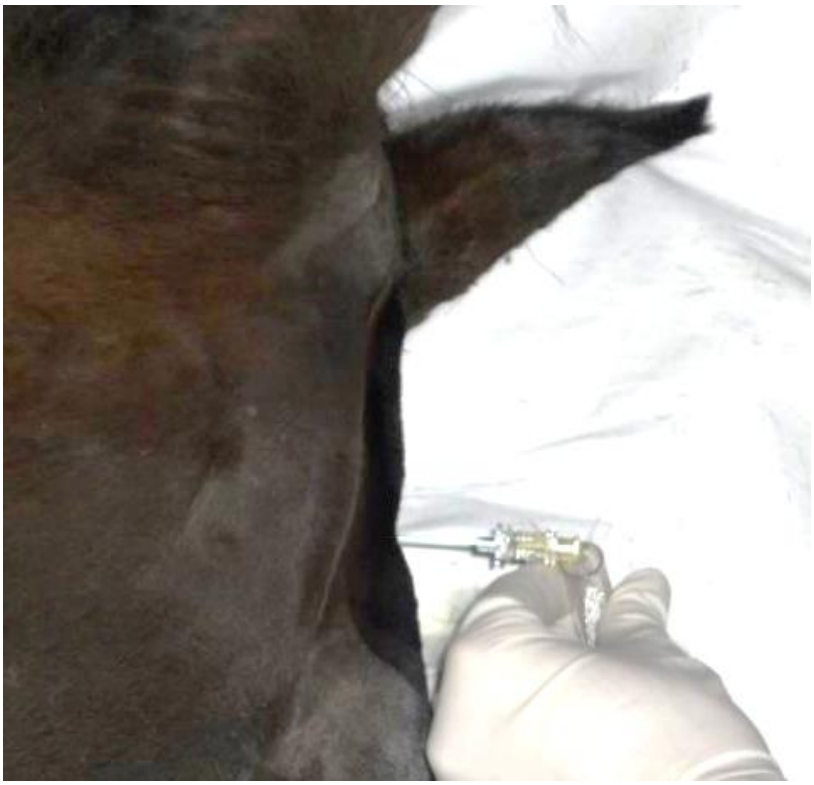


Figure 10. Cerebrospinal fluid collection at atlanto-occipital sampling site

CSF was first analyzed macroscopically for color and turbidity by placing it in front of a white paper. Samples were centrifuged and stained with Wright–Giemsa within 30 minutes after collection and another sample from each horse was air-dried after cytocentrifugation and kept for further evaluation. Cytological analysis was performed within 6 hours in all cases. Protein content was measured using an ultrasensitive method, spectrophotometry (Olympus AU400, Beckman Coulter, Hamburg, Germany) and other biochemical parameters like the activities or levels of aspartate aminotransferase (AST), alkaline phosphatase (ALP), glucose, lactate, urea, creatine kinase (CK), lactate dehydrogenase (LDH), sodium, potassium, calcium, chloride, inorganic phosphate, and magnesium were determined spectrophotometrically by the use of commercially available test kits with a chemistry analyzer (Olympus AU640, Beckman Coulter, Hamburg, Germany).

In each case, we attempted to characterize the virus using peripheral blood leukocytes or brain and spinal cord samples by virus isolation, nested reverse transcriptase polymerase chain reaction (RT-PCR), real-time RT-PCR and sequencing techniques, as described previously (Kutasi et al., 2011).

In case of two euthanized horses, histology and immunohistochemistry examinations were carried out, after post-mortem dissection. Histology samples were taken from the medulla oblongata, pons, cerebellar cortex and cerebral cortex. Representative tissue samples of the central nervous system (CNS) were fixed in 10% neutral buffered formalin solution and processed for histology. Tissue sections were stained with hematoxylin and eosin (HE).

The virus neutralization (VN) test was run in 9 of 13 WNND cases and on all control CSF samples as described by Nagy et al. (Nagy et al., 2019).

When analyzing our results, first we evaluated the normality of our data, i.e. we determined whether parametric or non-parametric statistics should be used. The majority of the data obtained from the WNND cases did not seem to follow normal distribution, and their mean, mode, and median were not close to being equal; hence, the t-test based comparison of the means of the diseased and control groups was not feasible. Instead, we opted to establish reference ranges (95% prediction intervals) based on the control groups and counted the number of cases for each variable that fell outside this range.

For the WNND cases we also noted the minimum and maximum values and established the median for each parameter. We used the Wald method and Fisher's exact test to evaluate which measured variables were predictors of the outcome. Finally, Fisher's exact test was applied to establish the relationship between the measured parameters (IBM SPSS Statistics 20 Documentation, United States).

Results

During the study period we sampled 13 horses affected with WNND. All CSF sampling procedures were performed without any complications. The horses of the control group recovered quickly from the anesthesia without any sequelae. Three horses from the WNV group were euthanized right after the sampling procedure, and three others were euthanized on humane grounds within the next 5 days. Seven horses survived the neuroinvasive disease, of which five had no sequelae. During PCR analysis, all premortem peripheral blood leukocyte samples tested negative for WNV and all post-mortem CNS samples tested positive. Attempts at virus isolation were successful in two cases on post-mortem CNS tissues. In all six cases where the disease was identified by means of PCR or virus isolation, the lineage 2 strain sublineage 2d was identified (Table 5).

Slight to moderate lymphocytic inflammations localized at the caudal brainstem, mainly at the pons and medulla oblongata, were observed. Central chromatolysis, cytoplasmic swelling, or cell shrinkage characterized neuronal degeneration in the most severely affected areas. The inflammation manifested in single-lined, perivascular lymphocytic infiltrations (perivasculitis), mild, focal glial cell proliferation, and mostly localized perivascular and scattered neuronal death. With a specific immunohistochemical reaction to West Nile virus antigens, viral antigen conglomerates could be detected in the nerve tissue according to the sites of inflammatory lesions. Figure 11-13. display the histology and immunohistochemical findings. We examined only brainstem samples; no samples from the spinal cord were taken.

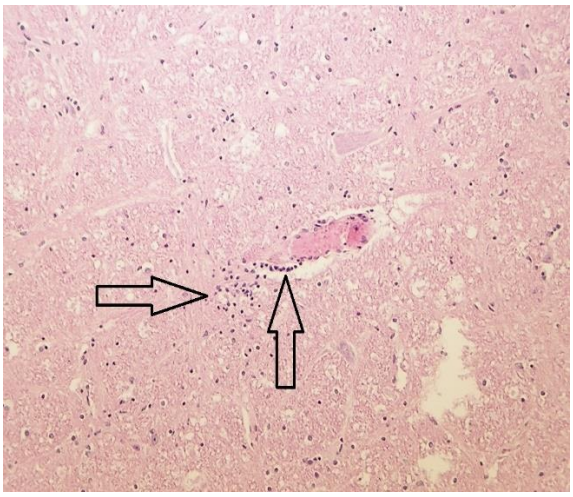


Figure 11. Discrete lymphocytic vasculitis suggestive of viral infection (vertical arrow) and perivascular glial cell proliferation (horizontal arrow) in the caudal part of the brainstem. H.-E. 400x

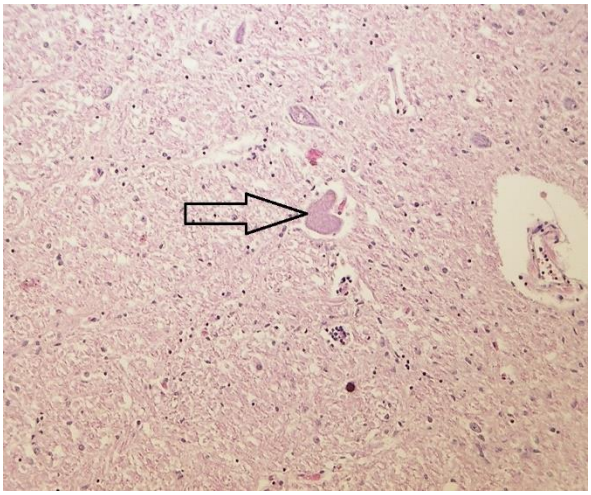


Figure 12. Neuronal degeneration (swelling, deformation and lysis of the cell nucleus) (arrow) in the caudal part of the brainstem. H.-E. 400x

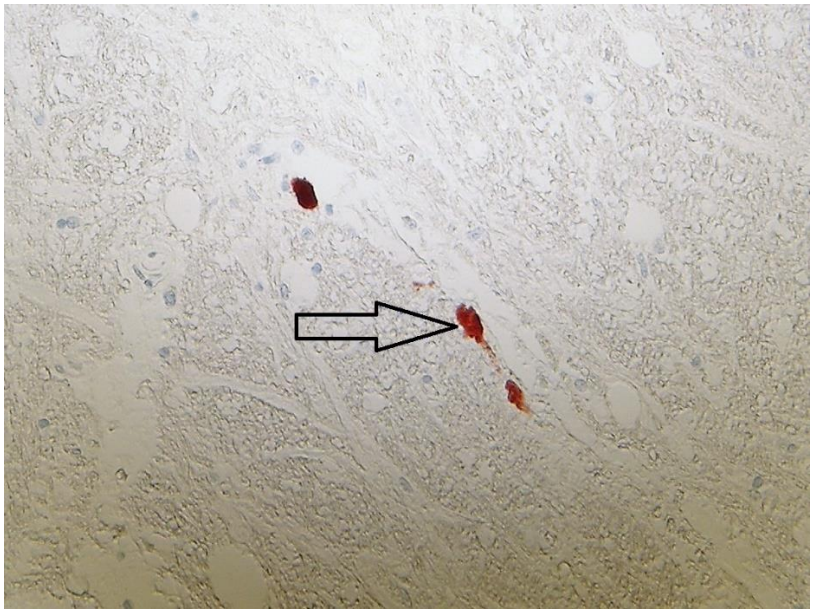


Figure 13. West Nile virus antigen conglomerates in the caudal part of the brainstem. Immunohistochemical reaction, 400x

The VN test of the CSF was negative in all control horses and in 5 patients, and positive in 4 WNND horses (Table 5). All the CSF-positive horses and one negative animal survived and in all the other negative horses the disease was fatal.

On macroscopic examination, the CSF was found to be transparent and non-turbid in all control animals and in nine WNV cases and slightly hazy in six WNV cases. Cytological analysis revealed normal cell counts within the reference intervals with exclusively small and large mononuclear cells in all control samples and in samples from three diseased horses. Out of all 15 samples of diseased horses, four had mononuclear pleocytosis and eight had neutrophilic pleocytosis.

We found moderately high total protein and mildly low albumin levels in 53.8% and 75% of cases, respectively. ALP activities, lactate and glucose concentrations were out of the reference ranges in 91.6%, 70% and 50% of the affected horses, respectively. Table 6. summarizes our further findings.

We also determined whether any of the measured variables were good predictors of the outcome (death/survival) of the disease. A noteworthy finding was that none of the six horses with elevated glucose levels survived the disease (0/6; ≤0.36; modified Wald method with 90% CI) and all of the 6 horses with normal glucose levels survived (6/6; ≥0.64; modified Wald method with 90% CI). The dependence of the outcome on the glucose level was also verified with a Fisher's exact test (2-tailed; p=0.0022).

In the two WNV cases where samples were collected using both atlanto-occipital and lumbosacral sampling methods, the results differed depending on the location. The atlanto-occipital CSF sample from horse no. 5 yielded negative results on cytology, the sample contained normal protein but high glucose and lactate levels. The sample obtained by lumbosacral puncture showed lymphocytic pleocytosis, high protein, and higher glucose and lactate levels. In horse no. 4, the lumbosacral sample showed lymphocytic pleocytosis, while the atlanto-occipital sample had more neutrophils with higher protein content, CK, LDH, and AST activities, and lower urea. Glucose levels were similarly high in both samples. None of these horses survived.

Table 6. Results of cerebrospinal fluid analysis comapraed to prevoiously published data [a,b,c]

	Inflammatory proteins (total albumin)	Albumin (mg/L)	Total protein (m/L)	AST (IU/L)	ALP (IU/L)	GGT (IU/L)	Glucose (mmol/L)	Lactate (mmol/L)	CK (IU/L)	LDH (IU/L)	Urea (mmol/L)	Na (mmol/L)	K (mmol/L)	Ca (mmol/L)	Cl (mmol/L)	P (mmol/L)	Mg (mmol/L)
Literature references			5-100[a] 20-124[b] 20-80[c]	15-50[a] 0-16[b]			1.67-3.89[a,c] 30-70% of blood glucose[b]	1.92-2.3[b]	0-8 [a,b,c]	0-8 [a, b]		140-150 [a,c]	2.5-3.5 [a,c]		9-123 [a,c]		
Our reference range (based on the control group)	0-56	10-50	32.16–75.55	6.0-14.0	0.1-3.5	0-4.0	2.54-3.81	1.89-3.07	0-4.6	14.7-44.1	4.6-8.8	140-151.8	2.8-3.1	1.13-1.41	113-128.2	0.02-0.38	0.53-1.34
Median	28.5	20	45.15	10.25	1.7	0.9	2.875	2.455	2.675	26.2	6.7	144.45	2.9	1.26	122.3	0.295	0.885
Minimum-Maximum	0-110	0-27	40.4-210.0	7-20.9	1.2-14.1	0-2	2.52-5.8	1.93-6.64	1.4-63	10-111.6	3.5-6.7	123-148.4	2.7-3.1	1.11-1.34	109-147	0.2-0.85	0.51-1.75
WNND group median	64	10	90.6	11.6	9.0	1.1	3.21	3.85	8.0	43	4.7	140	2.9	1.24	119	0.32	1.03
Number of WNND horses with normal values	4	3	6	6	1	12	6	3	5	6	9	8	11	6	7	9	7
Number of WNND horses with abnormal values	8	9	7	6	11	0	6	7	7	6	3	4	1	0	5	3	5

Note that inflammatory protein is a calculated value based on the total protein and albumin levels.
WNND: West Nile neuroinvasive disease; AST: aspartate aminotransferase; ALP: alkaline phosphatase; GGT: gamma-glutamyltransferase; CK: creatine kinase; LDH: lactate dehydrogenase; Na: sodium; K: potassium; Ca: calcium; Cl: chloride; P: phosphate; Mg: magnesium.
[a]Mayhew et al. (1977) ; [b]Andrews et al. (1990) ; [c]McWillimas (2002)

Discussion

This is the first paper that describes in detail the characteristics of the CSF of horses with WNND caused by a lineage 2 strain and proposes some clues for prognosis and pathogenesis.

The reference ranges set by our control group were consistent with those in previous studies (Cowell & Tyler, 2002; Mayhew et al., 1978), except for lactate concentration that was slightly higher and LDH activity that was moderately higher in our reference group. This could be attributed to the different methodology used by our laboratory.

In previous studies involving human patients, the CSF findings of patients with WNND were non-specific and included pleocytosis (neutrophil or lymphocyte predominance) with elevated protein and normal glucose levels (Tyler et al., 2006). Our findings are very similar to these findings, except that we have found high glucose levels in non-surviving patients. On the other hand, previous reports in horses most commonly found mononuclear pleocytosis with lymphocytic predominance (Wamsley et al., 2002). Although the WNV disease was caused by lineage 1 strains in those cases and lineage 2 strains were responsible in the present report, lineage differences are not likely to be the reason behind these discrepancies. Other studies on human CSF also showed variable data, where patients did not present with the typical lymphocytic pleocytosis, often alluded to when discussing viral meningitis and encephalitis, but rather, most presented with a cerebrospinal fluid neutrophilia (Crichlow et al., 2004; Rawal et al., 2006; Tyler et al., 2006). Previous studies with mice suggest that neutrophils are the predominant immune cells that are initially and rapidly recruited to the sites of infection with WNV (Lim et al., 2011). According to a study involving human patients (Rawal et al., 2006), the mean total leukocyte counts and mean neutrophil fractions were greater in samples collected within the first three days of the clinical signs than in those collected beyond day 3. Sampling time might also be responsible for the different findings. The samples were collected from most of our horses relatively early in the disease process (in all horses within 5 days and in 8 within 3 days of the onset of clinical signs). According to an early study about CSF in equine patients, the presence of neutrophils strongly suggests meningitis (Beech, 1983). Different severity and involvement of the meningeal inflammation could also explain the variable cytological findings. In another study, it was found that older patients with WNV were more likely to have neutrophils in their CSF (Jordan et al., 2012). The average age of our patients with high neutrophil numbers was 8 years (4–13 years) and of those with mononuclear pleocytosis was 6.6 years (4–9 years). Furthermore, neutrophil-related proteins were found at higher levels in the CSF of patients with WNND, underlining the likely key role played by neutrophils in the development of the inflammatory response and brain damage (Fraisier et al., 2014). We have also found that CSF neutrophilia is more likely to be found in parallel with high

protein content. The measured data indicated that neutrophilic pleocytosis in the CSF was more likely in cases with high total protein content (Fisher's exact test; 2-tailed; p=0.1026). In case when the calculated P value was ~0.1, our data could not be regarded as a definitive proof of the dependence of the classification. However, we decided that the value was low enough so that the observed dependence was worth pointing out to the reader. The increase in protein could reflect the increase of endothelial cell permeability, which would indicate that the blood–brain barrier is damaged in WNV patients. The level of albumin was lower in most cases and that of total protein was high, suggesting the presence of increased inflammatory proteins like globulins in the CSF of diseased animals. It is difficult to distinguish whether viral or immune-mediated neuronal injury is more significant in a disease process, and probably because of the low sample number, we were unable to demonstrate any relationship between the protein levels, the presence of neutrophils and the outcome.

We detected normal or high CSF glucose concentrations similar to the findings of a study on seasonal human epidemic WNV meningoencephalitis (Rawal et al., 2006). Cerebrospinal glucose concentrations might reflect changes in blood glucose, which could be elevated because of critical illness causing a stress response (Andrews et al., 1990; Seehusen et al., 2003). Human and animal studies suggest that this is not a benign condition and that stress-induced hyperglycemia is associated with a high risk of mortality (Capes et al., 2001; Guo et al., 2014). Increased lactate levels were found in most of the cases as well as increased LDH levels in half of the samples in the WNV-affected group. L-lactate is formed during normal anaerobic glycolysis by interconversion from pyruvate via the actions of LDH. Lactate concentrations in the CSF largely represent its production by the brain but it is also increased in case of low glucose concentrations to meet the energy requirements via the anaerobic pathway (Irani, 2009). Hypoglycemia was not seen in our cases. Increase in CSF lactate concentration reportedly occurs with bacterial infections but not with non-septic meningitis (Schwarz & Piercy, 2010). On the other hand, an increase in the level of CSF lactate also occurs with any condition that results in reduced brain oxygenation and/or increased intracranial pressure. CNS tissue hypoxia could be the result of inflammatory processes secondary to WNV infection. The potential influence of ketamine use on brain energy metabolism was considered, but when looking at the results of the control group, this was excluded. Ketamine has been shown to increase brain lactate concentration in rats and glucose levels in mouse brain (McLoughlin et al., 1987). In the control group, horses undergoing general anesthesia had slightly lower glucose (2.89 ± 0.186 vs. 3.11 ± 0.445) and lactate (2.37 ± 0.237 vs. 2.81 ± 0.729) concentrations than horses sampled standing.

As most enzymes are relatively large molecules, there is very little diffusion of enzymes across the intact and normal blood–CSF barrier and increased activities of the enzymes in the CSF are assumed to be facilitated by the cells within the CNS (Furr & Reed, 2015). The potential sources of increased enzyme activity in the WNV group horses are inflammatory cells or the damaged nerve cells and myelin. Recently, it has been shown that WNV induced the expression of interleukin-1ß, -6, -8, and tumor necrosis factor-α, where neurons were one of the potential sources of pro-inflammatory cytokines, and these pro-inflammatory mediators were one of the main factors driving WNV-induced neurotoxicity, cell death and CNS tissue damage (Lim et al., 2011). Based on previous histological findings of WNV encephalitis, namely perivascular inflammation, microgliosis, variable degree of necrosis and loss of neurons, it is less likely that the source of increased enzymes is secondary to a damaged blood–brain barrier or blood–CSF barrier and increased leakage from the plasma.

The most reliable parameter was the increase in the activity of the alkaline phosphatase enzyme. The CSF of patients without neurological disorders contains little or no activity as it was also shown in our control group (Bowers & McComb, 1975). According to a previous human study, the CSF alkaline phosphatase activities of patients with meningitis and other neurological disorders varied in direct correlation with the number of polymorphonuclear leukocytes present and with the protein concentration (Bowers & McComb, 1975). We could not demonstrate a clear relationship between the number of neutrophils, the level of protein, and alkaline phosphatase activity. The reason behind this might have been the low sample number.

There is surprisingly very little information about urea levels in the normal CSF although an increase would be significant in differentiating uraemic encephalopathy. Since urea is readily diffusible, the urea levels should be parallel to those found in the serum (Irani, 2009). Decreased urea levels in some of our patients' samples could be secondary to reduced hepatic synthesis of urea from ammonia in case of severe systemic disease. None of our patients had increased urea concentration in their CSF.

The electrolyte composition of the CSF has been sparsely reported, but in general, the concentrations of sodium and chloride in the CSF are similar or slightly higher, those of potassium in the CSF are similar or slightly lower, and those of magnesium in the CSF are slightly higher than those in the serum (Cowell & Tyler, 2002; Mayhew et al., 1977). Our reference ranges based on the control group were concordant with the results previously published. In some of the WNV-affected horses, low sodium and increased magnesium

concentrations were detected. These electrolyte abnormalities can originate from the cellular damage in the CNS, where intracellular solutes may leak out of the cell because of increased membrane permeability and lead to the redistribution of sodium and increased magnesium. In studies involving humans, phosphate was found in the normal CSF at levels of 50–60% of the expected serum concentration and it has also been observed previously that inorganic phosphate concentrations in the CSF increase in direct proportion to total CSF protein levels (Irani, 2009). Although we found increased inorganic phosphate concentration in three cases, a similar relationship could not be demonstrated.

The histological lesions and clinical signs observed in the affected horses were similar to previously described cases of natural equine WNV infection, where a detailed pathological examination was carried out. Lesion morphology and distribution were typical of polioencephalomyelitis, with prevalent involvement of the lower brainstem (Cantile et al., 2001).

When we collected samples from both lumbosacral and atlanto-occipital sites from the same patient, we obtained different results. This can be attributed to the different locations of the more severe CNS damage causing more significant alterations in the sample collected from the site closer to the area of damage. Although there is a synchronous appearance of WNV at many sites in the brain and spinal cord, and pathological alterations can be detected in many parts of the CNS, the severity of these damages can differ, also reflected in the clinical signs and disease progression. On the other hand, the values of certain parameters differ depending on sampling sites even in healthy horses because of differences in the blood–CSF permeability and flow rates between the atlanto-occipital and lumbar regions (Vernau et al., 2008). We must note that although neutralization test of the sera to detect specific antibodies is the gold standard of ante-mortem diagnosis of WNV infection (Long & Barr, 2017), the same does not apply to the CSF. A negative VN test on the CSF does not rule out WNV infection. It is of interest, but statistically not examined because of the low sample number, that horses without detectible neutralizing antibodies in the CSF were less likely to survive. Increased risk for fatality in these cases might be explained by the weak or absent intrathecal antibody production.

Based on our results, CSF sampling facilitates the differential diagnosis. Contrary to our findings in horses with WNND, CSF samples from patients with equine herpesvirus myeloencephalopathy typically show xanthochromia and have increased total protein

concentrations (principally albumin) without a concomitant increase in cell count (Pusterla et al., 2009). Horses with rabies have slightly elevated total cell count, with a predominance of lymphocytes and occasionally increased protein concentration in their CSF (Green et al., 1992). So far it was believed that neutrophilic CSF analysis in patients with suspected viral encephalitis was suggested to be typical of Alphaviruses, rather than WNV (Wamsley et al., 2002). Neutrophilia might be misleading in some cases but Alphaviruses causing neurological disorders in horses, like Eastern, Western and Venezuelan equine encephalomyelitis viruses, are non-endemic in Europe. CSF changes resulting from the migration of parasites show xanthochromia, an increase in protein concentration, and a slight mononuclear, eosinophilic or, in terminal phases, neutrophilic pleocytosis (Darien et al., 1988; Mayhew et al., 1978). These findings except eosinophilia and xanthochromia might be overlapping. Bacterial meningitis causes xanthochromia, moderate to marked suppurative inflammation, and increased protein and lactate levels in the CSF (Toth et al., 2012). Cervical stenotic myelopathy does not result in obvious alterations of the CSF.

CSF analysis provides a general index of neurological health and often provides evidence of the presence of a specific disease. Similar to a complete blood count, CSF analysis has reasonable sensitivity but low specificity. The CSF findings of patients with WNND are nonspecific and variable and possibly depend on the age of the patient, the sampling time, the site of sampling and previous treatments. Neutrophils likely play a role in the development of inflammatory response and brain damage, but further examinations would be required to fully elucidate their role in the pathogenesis of WNND. Increased enzyme activities and changes in the electrolyte concentrations reflect CNS cellular injury rather than blood–brain barrier damage. A higher sample number would be required to demonstrate the relationship between inflammation, CNS damage, and changes of the CSF parameters. Although elevated glucose levels reliably predicted the outcome, based on the observation that all non-surviving patients had high plasma glucose levels as well, these results might be secondary to increased plasma levels and reflect general stress or serious illness rather than any CNS pathophysiology. Based on all these findings, examination of the CSF is most useful when the results are correlated with the history, the clinical findings and the results of ancillary laboratory tests.

Study III

Treatment of West Nile neuroinvasive disease in horses at their home premises

Materials and Methods

Case one was a 10-year-old, Draft breed, chestnut mare, which was kept in the suburban region of Karcag. The second case was a 2-year-old Nonius, a black mare in Balmazújváros. Both horses were used for hobby purposes and were under the everyday supervision of their owners. Stables were located in inhabited areas of the cities, and none of the horses has left their home premises. In both cases, native blood samples were sent to the Hungarian National Food Chain Safety Office (NÉBIH) for official testing of West Nile Virus infection. Laboratory testing was conducted according to the OIE (WOAH) guidelines, and both patients tested positive for the presence of IgM antibodies during the ELISA assays.

Results

Only the Nonius mare had any vaccination history and received a vaccine against equine influenza and tetanus toxins, but none of the horses were vaccinated against West Nile virus. In both cases, the onset of clinical signs was 1-2 days before the veterinarian's notification. First, only mild signs appeared which were hard to see at once, but their condition was rapidly deteriorating. Observed clinical signs are summarized in Table 7. Detailed clinical examinations were carried out on both horses, according to international literature and recommended protocols (Furr & Reed, 2015; R. S. Porter et al., 2011). During the disease, clinical parameters were physiological; both horses had appetite and were able to take up food alone. The first observed sign was the incoordination of the gait, which was more conspicuous on the hind extremities. Asymmetrical ataxia was scored according to the international recommendation, and the unstable movement (2/5) has rapidly deteriorated to collapse and recumbency (4-5/5) (Furr & Reed, 2015).

Table 7. Observed clinical signs during WNND

	Case 1	**Case 2**
Onset of clinical signs	August 2016	September 2016
Pulse rate/minute	28-36	30-38
Respiratory rate/minute	~11	~14
Highest rectal temperature	37, 5	38,7
Ataxia	deteriorating , 4/5	deteriorating , 4/5
Type of ataxia	asymmetric (right hind limb)	asymmetric (right hind limb)
Paresis	on all limbs	right hind limb
Recumbency	total recumbence	collapse, periodical recumbence
Hyperesthesia	whole body	whole body
Dysphagia, facial nerve paralysis	no	no
Urination, feces	physiological	physiological

In case two, the horse became hyperesthetic and collapsed after a mild stimulus. Following the collapses resulting from hypersensitivity, the animal could be set up simply with less assistance, did not remain recumbent for a long time, and its consciousness was maintained throughout. The horse was held in a standing position by a rope, passed through the halter and a special knot placed on the tail (Gozalo-Marcilla & Ringer, 2021). The tail knot used in this mode of assistance and its installation instructions is illustrated in Figure 14. The initial severe signs began to improve within a few days, and the horse recovered from the disease without any residual signs. We consider it to emphasize that sometimes recovery from this kind of disease can depend on techniques that are simple to do at home.

Figure 14. Procedure for placing a tail rope to support the hindquarters or assist a horse to stand

In case one, shortly after the onset of ataxia the horse became recumbent and was unable to stand up without special aid. In this case, referral to an equine clinic should have been the first option, but the owners have rejected the transportation of the patient. The owners have been informed in detail, about the potential danger, complications, and poor prognosis, but did not want to euthanize the horse. A homemade sling was introduced from a girth, and with the help of an agricultural truck, the horse was pulled up to a standing position. The procedure took place at the stable yard every 8-12 hours. During lateral recumbency, the girth was applicated under the horse's chest and inguinal region with the help of loops and hooks and the horse has been lifted to sternal recumbency first. The horse maintained consciousness during the whole weakness and was ably encouraged by the owners to make an effort and this power has been added to the lifting force of the machine. The time spent in the sling was continuously increased each time. By the third week after the onset of the first signs, the horse was already able to stand approximately 60 minutes with the sling support, and around the fourth week, he was able to stand up without the help of the machine. The procedure was carried out with the work of 3 to 4 people, under the supervision of a veterinarian. After two months, the horse recovered from the disease, but hindquarter paresis consisted of a residual sign, which severity did not decrease with time.

Cases mentioned in this study, were treated at their home premises due to financial limits. Administered medication was based on anti-inflammatory, antioxidant, and fluid therapy, as well as on pain relief. In case one, the recumbent horse has received A, D, E, K vitamins and additional selenium, in injections of Norovit 4 Komplex A.U.V. inj., (Norbrook, 20-30 ml/horse, IM.) and Soluselen inj. (Phylaxia-Pharma, 1 ml/ 10 kg, im) and repeated in the oral form twice, 3 days apart. Pain relief and anti-inflammatory treatment were performed with non-steroidal anti-inflammatory drugs (NSAID) for 3 days, with an active ingredient of flunixin meglumine (Norflunix 50mg/ml A.U.V., Norbrook, 1.1 mg/kg, iv) and with phenylbutazone (CP-Phenylbutazon 20% A.U.V., CP-Pharma, 4.4 mg/kg, iv/po). One dose of short-acting dexamethasone with an approximately duration of 24 hours was also administered. Fluid and electrolyte supplementation was performed by intravenous infusions during the first 3 days, supplemented on the first day by an infusion of Duphalyte containing glucose, dextrose, vitamins, and amino acids. In the case of the sick horse in Balmazújváros, the treatment protocol was similar; however, depending on the degree of signs, shorter therapy was sufficient.

Discussion

The disease of the horses, included in this case study, occurred between August and September. This period is during the seasonal time of West Nile Virus infections, typical for Hungary. Continuous climate change and the increasing prevalence of vector mosquitoes are allowing infections to appear earlier each year. Based on this fact, in the case of horses with neurological signs during the summer and autumn months, the differential diagnostic list should also be considered primarily for WNV infection (Kutasi et al., 2011). In 2016 and 2018, the number of human and equine WNV infections increased significantly, compared to previous years (ECDC). There are commercial vaccines available for equines for the prevention of the neurological form of WNV infection. A double dose of injection 3-4 weeks apart, secure a good immunity, and revaccination should be administered approximately 1 month prior to the season. In recent years, a large number of samples have been taken to detect West Nile Virus infection due to extensive information of practicing veterinarians, but it is still assumed that a lot more infections stay undetected. In both cases, high levels of IgM antibodies were detected in ELISA tests, which proved the recent infection of the horses (Beck et al., 2013).

Neurological signs experienced during WNV infection, are not characteristic and may also occur in other infectious diseases. When animals develop neurological signs, rabies should be considered one of the major zoonotic viruses. Both horses were handled strictly in disposable

gloves only. The current epidemiological situation in Hungary, the nature of the acute clinical signs, and the lack of injury to the horses did not justify the establishment of a suspected rabies status, so it was not ordered officially either. Given that the horses were under constant human supervision, it can be assumed, that the owners detected the primary symptoms as early as possible. Among other pathogens, Borna disease does not occur in Hungary, and the chance of developing anaplasmosis was low, as the horses were not let to pastures and the owners did not notice any tick bites. The signs during WNND are similar to that caused by Equine Herpesvirus, although other syndromes, particularly the lumbosacral spinal cord region, are often associated with urinary incontinence and, less commonly, fecal excretion, as well as decreased tail tone (Furr & Reed, 2015). No urinary or fecal retention was observed in the animals described in our study. The horse in the sling usually urinated and passed feces in a standing position. Ataxia typically affects symmetrically the hindquarters in Equine Herpes Virus infection, and it rather appears asymmetrical in case of WNV infection.

If all other diseases from the differential list, were to be ruled out, several other serological, biochemical, and molecular biology assays, feed analysis, possibly muscle biopsy, cerebrospinal fluid examination, and X-rays would have been necessary (Bowen et al., 2006). In the present cases, the diagnostic tests had financial constraints, and since the WNV IgM serological result was obtained within days, further diagnostic tests became no longer necessary. In horses, mainly NSAID medicines are used to reduce inflammation and for pain relief. According to the literature recommendations, in both cases, flunixin meglumine (1.1 mg/kg, iv) and phenylbutazone (4.4 mg/kg, iv/po) were administered 3 days after the onset of clinical signs (Furr & Reed, 2015; Gardner, 2011; Nout & Reed, 2010). Further administration of NSAIDs in a recumbent, possibly dehydrated animal may lead to severe renal insufficiency, and therefore relatively short-term treatment was used (Gardner, 2011). Treatment with a short-acting glucocorticoid in this kind of disease is questionable. A single dose of dexamethasone (0.06 mg/kg, iv/im) has also been used in the treatment of the recumbent mare in Karcag. It is also mentioned in previous publications that in the case of recumbency in horses, the use of dexamethasone or methylprednisolone can be required (Furr & Reed, 2015). Corticosteroids may play a role in reducing cerebral edema through neuronal and vascular membrane stabilization, and the anti-inflammatory effects of dexamethasone and methylprednisolone are effective (Furr & Reed, 2015). Experiments in dogs suggest, that in addition to their beneficial effects, the immunosuppressive impact may increase viremia, which may result in increased mortality (Bowen et al., 2006).

In addition to fluid therapy, dimethyl sulfoxide (DMSO) (1.00 g/kg, iv) therapy could have been useful to reduce the edema and inflammation that has developed during the disease (Nout & Reed, 2010; R. S. Porter et al., 2011). Adjunctive therapy with mannitol infusion (0.25–1 g/kg, iv) and intravenous furosemide (0.5–1 mg/kg, iv) every 6 hours would have been possible in case of cerebral edema (Sejvar, 2014). The practical use of interferon-alpha (INF-α), as well as immunoglobulin therapy in horses, is not yet widespread (Furr & Reed, 2015). To prevent bacterial co-infections, the use of antibiotics may be warranted in the treatment of the disease. However, based on the general condition of the horses in the present study, the use of systemic antibiotics was not necessary. In the case of an immunocompromised animal, the development of pneumonia has a high risk, so regular monitoring of the respiratory system and the prophylactic treatment with antibiotics is essential in a recumbent horse. In present cases, no respiratory signs were observed in the control studies, and rectal body temperature varied within the physiological range, so antibiotic treatment was not warranted. In the case of the recumbent horse, only minor lesions developed on the skin surface, which was also shown to be responsive to topical treatments, and in addition, regular set-ups and bedding replacements were important. To avoid pneumonia and other undesirable, sometimes fatal complications, it is especially important to pull up the recumbent horses as soon as possible and to treat skin injuries (Furr & Reed, 2015; Ishihara et al., 2010; Nout & Reed, 2010; Pusterla & Madigan, 2006). It is essential to assess the state of consciousness of the animal before any manipulation or examination, to avoid accidents and to be able to determine the treatments accurately. In accordance with accident prevention, the sedation of the horses may be required (Ishihara et al., 2010; Pusterla & Madigan, 2006). It can be a real difficulty to provide the appropriate equipment for the animals in these severe conditions. Veterinary product distributor companies offer special suspension devices for horses, which can be used to pull up recumbent horses also in stable circumstances. Figures 15 and 16 show two slings, which are used worldwide in equine clinics. The two best-known products are the Anderson Sling Support Device (ASSD) and the US Davis Large Animal Lift (LAL). The use of these slings requires financial effort, expert staff, as well as time, but with all of the mentioned support, it can be used in stable conditions as well.

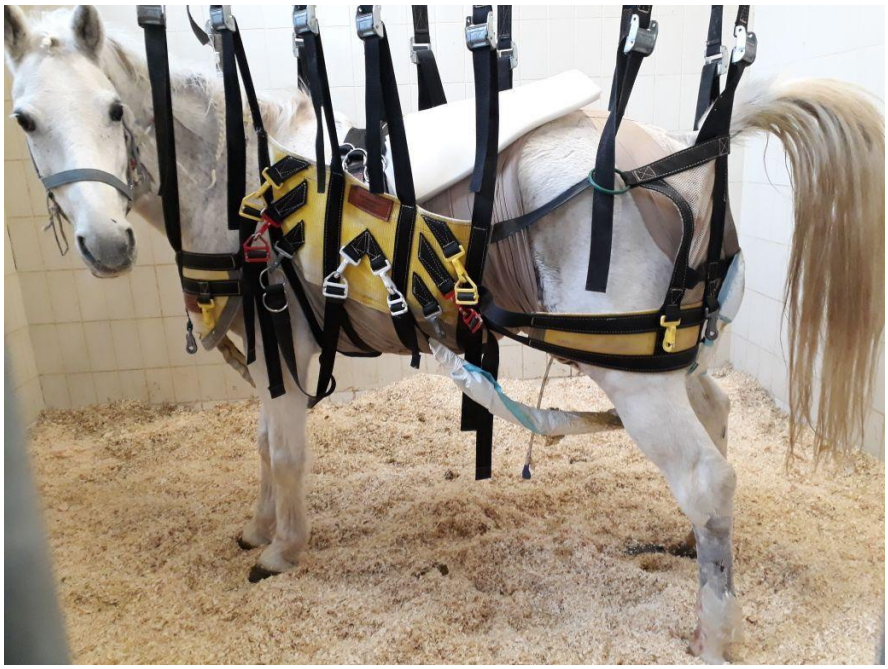


Figure 15. Professional equine Anderson sling (ASSD) at clinical circumstances. Photo was taken at the University Equine Clinic of Vetmeduni Vienna

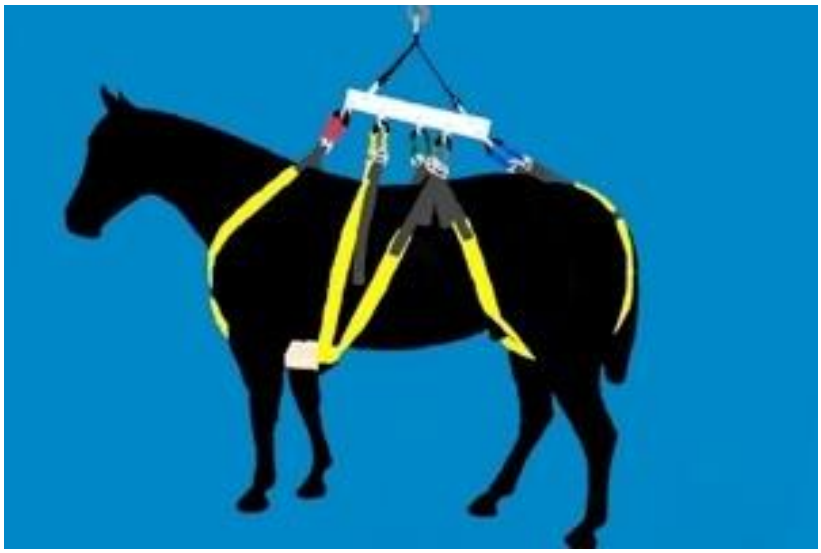


Figure 16. Picture of the US Davis Large Animal Lift
http://www.largeanimallift.com/horse/

To overcome financial constraints, simple suspension devices have also been developed, which can be manufactured at home (Pusterla & Madigan, 2006). In the cases discussed, it was not possible to transport the horses to a hospital, so under stable conditions, a sling for setting up the horse had to be made from the available equipment (Figure 17, Figure 18).

Figure 17. Sling made from high-tensile net girth

Figure 18. Sling made from carriage harness

There is limited opportunity for preparation for similar, urgent cases, both technically and in terms of staff, but with the right creativity and determination, the necessary conditions can still be provided. Before starting treatment, it is important to assess the attitude and commitment of the horse owners and staff, keeping in mind the needs of the sick animal. To avoid accidents and injuries, it is important to accurately assess the potential risks and benefits before treatment begins (Pusterla & Madigan, 2006). This type of procedure, with the right expertise, requires more time than financial resources. The opinion of an external, objective expert is also important, as people who are in constant contact with the sick animal, may judge the development of the disease emotionally biased. In order to avoid accidents, the lifting gear must be handled exclusively by a competent person with appropriate experience and following operating instructions. It is important to consider the weight, physique, and temperament of the animals when designing the homemade sling. Based on the construction of special suspension devices for horses, a sling that can be placed on a horse (for example, made of straps) and a part of lifting equipment (lifting machine, hand winch) must be a standard accessory for the suspension device.

Before treating any similar recumbent horse, it is important to inform horse owners about the dangers of the procedure, and its questionable outcome. It is important to seek their consent, even in writing. As with factory straps, several types of homemade suspension straps are possible. They can also be made by modifying a special horse harness (Figure 17), using wide spanifiers, or using a high-tensile mesh (Figure 18). The most important expected feature of the sling device is that it has both abdominal and chest suspensions. The straps should be as flat as possible and, in the case of long-term use, adequately soft (Pusterla & Madigan, 2006). When suspending the straps, it is important to try to distribute the load on each part of the body, which can best be ensured, for example, with a two-point lifting device (Figure 16, Figure 17).

To avoid accidents, when installing the harnesses, care should be taken to keep the staff behind the back of the lying horse, avoiding injuries caused by the horse's feet (Pusterla & Madigan, 2006). According to the recommendations, homemade hitches can be used for approximately 20-30 minutes each time in case of a recumbent horse, which is unable to stand alone in the sling. However, in the case of horses that can remain in the assisted standing position, the devices and slings can be used for up to 12 hours (Pusterla et al., 2009). In the case of the horse from Karcag, first, the animal was rotated every 8 to 10 hours, and then the horse was lifted with a suspension device moved by a two-fork agricultural machine (Figure 14). After the horse showed signs of fatigue, he was released back to a sternal position, during which food intake could be assured.

The treatment of the recumbent horse was performed by the authors alternately on-site, with continuous consultation. Changing the position promotes the optimal circulation of the skin in addition to the proper functioning of the lungs while reducing the chances of developing myopathy (Furr & Reed, 2015; Gardner, 2011; Nout & Reed, 2010). As far as possible, it is important to provide soft, dry bedding around the recumbent horses, thus reducing the development of any pressure sores, which occur primarily around the tuber coxae, tuber ischia, and the shoulder area.

The bedding should not be too deep, as it should provide adequate support when lifting the recumbent horse. With the use of blankets, we can reduce superficial skin damage and abrasions, which may be caused by the straw. Wet bedding can promote the development of cellulitis and decubitus (Gardner, 2011; Nout & Reed, 2010). The bedding was recycled at each lift, and the worn, dirty straw was changed to fresh. To protect the horse's head, a large blanket was also spread over the straw to help prevent superficial skin and ocular damage. The use of special head and eye protection is also common in equine hospitals. In addition to the strap installation, a special knot (Figure 11) placed on the tail and a rope passed through the halter can be used. Dysphagia can often occur in horses with nervous system disease. If there is no difficulty in swallowing, the lying horses should be fed and watered. Horses prefer to take up food in a standing position, so efforts should be made to set up the horses and feed should be lifted to the height of the horse's nose.

In the case of horses unable to swallow, the energy requirement should be provided by feeding through an nasogastric tube, or possibly intravenously, with appropriate infusion preparations. Good quality fibrous feed, minerals, and vitamin supplementation can ensure the needs of the horses. Continuous monitoring of fecal and urinary excretion and the provision of dry bedding are essential for a recumbent horse. Both horses were fed exclusively with hay, and concentrated feed was avoided. In the case of long-lying, immobile horses, laminitis can often develop, so monitoring the temperature of the hooves and the pulsation of the digital arteries at least every 24 hours is of paramount importance (Gardner, 2011; Nout & Reed, 2010). However, during continuous monitoring, none of the horses showed signs suggestive of laminitis. In both cases presented, proper circulation of the hooves was ensured by setting up the horses.

The standing position promoted the optimal functioning of the lungs, so there was no systemic inflammation. Adequate feed also reduced the chances of developing laminitis. Since we could not provide continuous veterinary supervision next to the horses, we informed the owners that if they notice signs of laminitis mentioned above on the horse, it would be necessary to cool the limbs, supplemented with additional NSAID therapy. Based on international literature and our own experience, we consider it important to emphasize that the high mortality rate from West Nile Neuroinvasise Disease (WNND) is due to euthanasia for humane reasons, rather than spontaneous deaths (Furr & Reed, 2015; Pusterla & Madigan, 2006). The cause of death can be a large number of secondary symptoms, and lifting of a recumbent horse can significantly increase the chances of survival. The case of the two horses discussed in this study also supports our observation that recumbency does not conditionally result in death even under stable conditions.

Study IV

Serum neutralizing antibody titers against a lineage 2 neuroinvasive West Nile Virus strain in response to vaccination with an inactivated lineage 1 vaccine in a European endemic area

Materials and Methods

Enrolment criteria and animals:

Blood samples were taken from 82 mixed-breed warmblood horses 7 and 14 days prior to the start of the study at a warmblood stud farm in Hungary to determine the serological status of the examined 82 horses and to be able to select seronegative animals for the vaccination study. These blood samples were tested for the presence of IgG antibodies against WNV with IgG enzyme-linked immunosorbent assay (ELISA), which screening tests were conducted at the Department and Clinic of Food Animal Medicine at the University of Veterinary Medicine, Budapest using ID Screen® West Nile Competition Multi-species ELISA (ID vet., Grabels, France). The ELISA test has a high sensitivity, but due to the possibility of the included monoclonal antibody cross-reactions with other Flaviviruses the test has a relatively low specificity. The fact, that cross-reaction may occur during the pre-screening ELISA test, does not influence our results, as the enrolment criteria require seronegative horses.

Thirty-two seronegative horses were randomly enrolled in the vaccination study of which 22 horses were allocated to the vaccine group and 10 retained as unvaccinated controls. Age of the 25 mares and 7 stallions/geldings was between 3 and 19 years old at the start of the study.

Study design:

Blood samples were taken from all of the 32 enrolled horses on Days 0, 49, 211, 393, 408, 576, 667 and 758 respectively and were tested for the presence of serum neutralizing (SN) antibodies against lineage 1 WNV and lineage 2 WNV strains. On Day 0, which was the start of the vaccination study, enrolled horses were tested with SN test and appeared to be seronegative for both lineages. The seronegativity was the main criteria for the enrolment into the study. General health observations were conducted on all horses throughout the study and detailed clinical examination was done at the time of all vaccination. Day 0, when the first vaccination was given, was scheduled in February out of the WNV season to avoid seroconversion due to natural infection. The primary vaccination consisted of two doses, the

first one followed by a second vaccination 4 weeks later (Day 28). The third vaccination was done on Day 393, corresponding to 1 year post second vaccination. Commercially available, ready to use Equip® WNV (Zoetis, Louvain-la Neuve, Belgium) vaccine was used. Each dose of vaccine was administered via a single intramuscular injection into the neck of the horses.

Laboratory analysis:

Blood was collected from the jugular vein of all horses into plain vacuum tubes at all sampling dates. Testing for the presence of serum neutralization antibodies against lineage 1 WNV and lineage 2 WNV were conducted at the National Reference Laboratory for Viral Zoonoses of the National Public Health Center, Hungary. West Nile Virus microneutralization assay was performed in accordance with the OIE Manuel (World Organisation for Animal Health, Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2013, Chapter 2.1.20. West Nile). In the recommendation of OIE, serum microneutralization is a suitable method for the monitoring of the infection-free status of a population, the prevalence of infection and the immune status in individual animals or in a population post-vaccination (OIE Terrestrial Manual, 2013). Serum samples were inactivated at 56°C for 30 minutes, and then twofold diluted in cell culture medium (Minimum Essential Medium Eagle; Sigma-Aldrich, Saint-Louis; USA). Serial twofold dilutions were prepared in 96-well tissue culture microtiter plates, using Eagle's Minimum Essential Medium (EMEM) in a 50 µl final volume. Each sample was titrated in duplicate, using two parallel rows of the microtiter plate. 50 µl of 100 TCID50 of both WNV strains were added to each well and incubated at 37°C for 90 minutes. By adding the virus suspension, the initial dilution of each sample became 1:10. 100 µl of Vero cell suspension (Cercopithecus aethiops kidney cell line) was added to the mixture of the diluted sera and standard virus suspension, resulting 200 µL final volume. Cell culture medium contained 5% inactivated fetal bovine serum (FBS; Sigma-Aldrich, Saint-Louis; USA). During the days of incubation microtiter plates were stored in a 37 °C incubator, at 5% CO_2 concentration. Microscopic evaluation of the cytopathic effect was carried out three, five and seven days after inoculation. Each well of the plate was thoroughly examined and considered seronegative if the degree of cytopathy was 50% or more.

Negative and seropositive human sera controls, negative cell controls and back titration of the virus suspension in at least three rows were also applied. Furthermore, in case of each serum sample, a serum control was prepared to detect any possible cytotoxic effect of the sample itself by adding 50 µl of cell culture medium instead of virus suspension. Serum neutralization antibody titers were measured against lineage 1 strain WNm1 (Molnár et al., 1973). Lineage 2 strain 578/10 used in our study was isolated from the brain of an infected horse during the

2010 epidemic in Hungary (GenBank accession number KC496015). Antibody titers of twofold serum dilutions were reported between 1:10 and endpoint titer. A titer of <1:10 was considered as negative, while a titer of 1:10 was evaluated as inconclusive result.

Data analysis:

Clinical observations were summarized by treatment group and over time in terms of the number and percentage with each clinical sign. Serum neutralizing titers were analyzed using a linear mixed model for repeated measures. An appropriate log transformation was applied to the data prior to being analyzed. The model included the fixed effects of lineage, treatment, lineage x treatment interaction, day, lineage x day interaction, treatment x day interaction and lineage x treatment x day 3-way interaction. Random effects included animal and error. Least squares means (LSM) and standard errors (SE) of least squares were estimated, and 95% confidence intervals were constructed. Back transformed least-squares means and confidence intervals were reported by lineage, treatment and day.

Ethical considerations

The study was approved by the Zoetis Ethics Review Assessment team under the permit number of 22.1/1606/003/2009.

The study was conducted according to the Standard Operating Procedure of Zoetis for Veterinary Medicine Research & Development (SOP-STUDY 026) which was based on EU Directive 2009/9/EC amending 2001/82/EC on the Community code relating to veterinary medicinal products.

The study was also conducted in full compliance with the guidelines of the Animal Experimentation Committee of Szent Istvan University, Faculty of Veterinary Science, Budapest, Hungary (50/2013 based on the license frame of 22.1/1606/003/2009).

Results

During the pre-screening ELISA test 14.63% of the examined 82 horses appeared to be seropositive. A total of 32 previously tested seronegative horses, were enrolled in the study on Day 0, with 31 horses completing the study on Day 758. One horse in the vaccination group had to be withdrawn from the study earlier on Day 408, because it was sold abroad. The withdrawn horse was not replaced in the study. During the health observations and clinical examinations on the vaccination days (0; 28; 393), there were no clinical signs in the enrolled equines.

Blood samples were analyzed using serum microneutralization (SN) tests for lineage 1 WNV and lineage 2 WNV strains. Most of the vaccinated animals had seroconversion against both lineages at Day 49 (three weeks after the second vaccination), when serum neutralizing antibody titers increased with the exception of 3 animals. These exceptional horses had a titer of 1:10, which is considered an inconclusive result. The yearly booster vaccination (third vaccination on Day 393) induced an anamnestic response in most of the vaccinated animals which was visible on Day 408, with the exception of 2 horses. Results of the exceptional two animals showed an increase in the SN titers, but the end titers of both lineages were only slightly positive and titer values did not exceed 1:80.

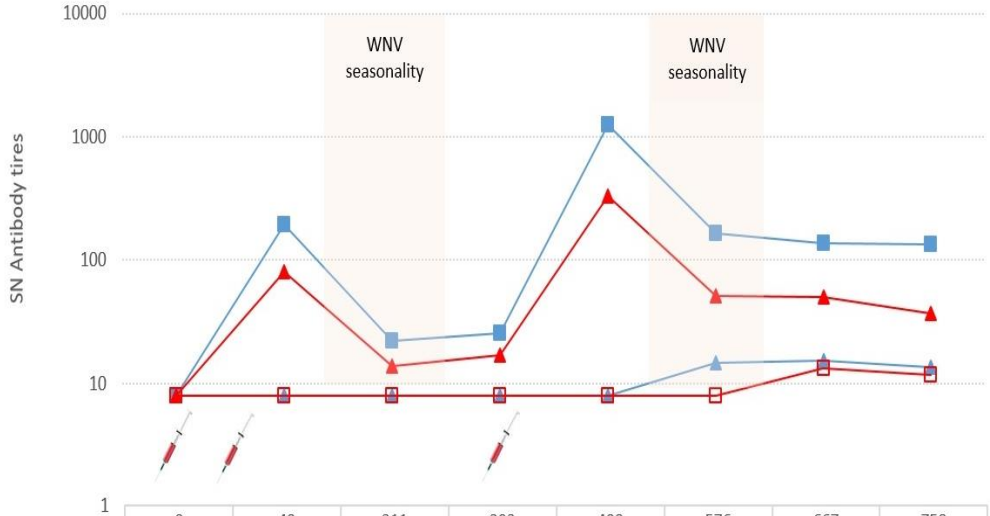
After Day 0 there was a significant difference between the vaccinated and control group in the serum neutralizing titers against both virus strains. Mean titers against the lineage 1 WNV strain were significantly higher ($P \leq 0.05$) in the vaccinated group compared to the control group on all time point besides Day 0. When comparing the control group to the vaccinated group, the mean titers against the lineage 2 WNV strain were significantly higher in the vaccinated group except for Day 211. Six months post primary vaccination (Day 211), there were no significant differences between treatment groups and neutralizing antibody titers against lineages 1 and 2 WNVs were below the threshold in 12 and 18 vaccinated horses, respectively.

Within each treatment group, a lineage comparison of mean SN titers was made. At all sampling points (except for Day 0), the vaccinated group had significantly higher ($P \leq 0.05$) mean antibody titers against WNV lineage 1 than lineage 2 strains. In the control group, a significantly higher mean SN titer against lineage 1 compared to lineage 2 was found on Days 576 and Day 758. There was no significant difference in means in the controls on any other time point, although individual animal results had differences in means between lineages on Day 667 in the controls. With the exception of Days 576, 667, and 758, the neutralizing antibody titers against both WNV strains were less than 1:10 in all unvaccinated horses. On these days, the least square means of SN antibody titers were above 1:10 in the vaccinated group.

After the booster vaccination on Day 408, the serum neutralizing antibody titers sharply increased, and approximately fivefold difference appeared compared to just after primary vaccination against both lineages 1 and 2 WNV strains. The titers showed some decline on Day 576, but at testing points on Days 667 and 758 no further significant decline could be observed as titers stayed relatively stable.

Three horses in the vaccinated group did not seroconvert after the primary vaccination and appeared to be poor responders. Although the control group did not receive any vaccination, five control animals on Day 576, a control horse on Day 667 and one animal on Day 758 revealed low but positive antibody titers against WNV. On Day 576, titers of the five mentioned horses were 1:20 against lineage 1, on Day 667 a horse had a titer of 1:80 against lineage 1 and 1:40 against lineage 2, while on the last testing day, the same horse had a titer of 1:160 and 1:40, respectively.

All least square means (LSM) of SN titers for lineage 1 WNV and lineage 2 WNV are summarized for each time point and each group in Figure 19.



	0 March	49 May	211 November	393 April	408 May	576 Oktober	667 February	758 April
unvaccinated: titre against lineage 1 strain	< 10,00	< 10,00	< 10,00	< 10,00	< 10,00	14,70	15,20	13,60
vaccinated: titre against lineage 1 strain	< 10,00	193,30	22,00	25,70	1240,30	166,00	135,80	134,50
unvaccinated: titre against lineage 2 strain	< 10,00	< 10,00	< 10,00	< 10,00	< 10,00	< 10,00	13,20	11,70
vaccinated: titre against lineage 2 strain	< 10,00	80,00	13,70	17,10	330,20	51,10	50,10	37,20

Figure 19. Plot of back transformed least squares means

Discussion

Vaccinated animals had significantly higher serum neutralizing antibody titers against both lineages of WNV compared to the control group up to two years after booster vaccination. Antibody titers produced were significantly higher against the lineage 1 compared to antibody titers against the lineage 2 strain. In a similar experiment in a South African endemic region, vaccination of horses with the same commercial vaccine Duvaxyn® WNV (Pfizer) stimulated the production of high titers of serum neutralizing antibodies against a neuroinvasive South

African lineage 2 WNV strain, although the peak and subsequent titers of serum neutralizing antibodies against the lineage 2 strain were lower than for lineage 1 WNV strain NY385/99 (Pearce et al., 2013). According to the South African and our study the vaccine developed against the New York 1999/VM2 lineage 1 strain more effectively induces SN antibody production against homologous lineage 1 strains than heterologous lineage 2 strains but cross neutralization occurs with lineage 2 strains. In previous studies the authors found out under experimental and field conditions that the antibodies produced by Equip® WNV vaccination allows for cross protection of horses against WNV lineage 2 challenge (Bowen et al., 2014) or from natural exposure (Chaintoutis et al., 2015). In conclusion, Equip® WNV vaccine stimulates the production of serum neutralizing antibodies against lineage 2 strain 578/10, a strain that was proven to cause neuroinvasive disease in horses in Hungary (Kutasi et al., 2011). The strain 578/10 has close relationship with lineage 2 strains isolated in outbreaks in other European countries like Greece, Italy and Germany (Bakonyi et al., 2006; Bouzalas et al., 2016; Calistri et al., 2010; Savini et al., 2012; Ziegler et al., 2019).

In this study, the first two doses of Equip® WNV vaccine were administered 28 days (4 weeks) apart, while in the previous study performed by our research group, these injections were given 21 days (3 weeks) apart (Joó et al., 2017). This difference in the vaccination protocol did not have an influence on the results, as both studies describe similar titer changes against both lineages 1 and 2. After primary vaccination serum neutralizing antibody titers against both lineages rapidly increased but dropped significantly by approximately 6 months post primary vaccination. These results had the same pattern as in previous studies (Davidson et al., 2005; Joó et al., 2017). Following a single annual booster, the serum neutralizing antibody titers increased to more than five-fold higher values than after primary vaccination and the decreased values on Day 576 were still significantly higher than those on Day 211. Our results are similar to the experiment of a previous study where one year after primary vaccination significant decrease in antibody titers were measured, while 12 months after the yearly booster all horses already had moderate-high level of antibody titers and this level could be maintained reliably with the second annual booster (Joó et al., 2017). These results suggest that in countries with seasonal WNV outbreaks like Hungary, where disease occurs seasonally between June and November (Kutasi et al., 2011) (ECDC 2018), timing of primary vaccination should be carefully planned possibly to late spring so that primary vaccination could be finished approximately 3-4 weeks before the suspected onset of the disease season.

An easier and more reliable protocol in this geographical region is to do primary vaccination after the season around November-December and give the booster right before the next season, approximately 6 months later around April-May. Regarding the result of our study, after primary vaccination with Equip® WNV, the first booster vaccination should be given earlier than a year to continuously maintain high antibody titers and immunity against WNV infection. During a previous Hungarian study on the duration of immunity induced by Equip® WNV vaccine, this pattern of recommended vaccination protocol has already been mentioned (Joó et al., 2017). If we follow the vaccination protocol of this recent study, an early spring vaccination may result in increase in SN titers, but the following decrease in the titer levels may coincide with the seasonal onset of the disease. Possibly because recently WNV outbreaks seem to appear earlier (Haussig et al., 2018) and stay longer (Joó et al., 2017),(ECDC), scheduling vaccination should always be adopted to the latest WNV surveillance data. According to our results, after the first annual booster, the titers stay sufficiently high in most horses all along the season and even longer, so afterwards a yearly booster offers reliable protection.

Figure 20. shows our recommendation for a new vaccination protocol in an affected area, where the season can widen due to environmental factors. The study was conducted in an endemic region of Europe where WNV outbreaks in humans and horses were reported during the study period (ECDC), so natural WNV and other endemic flavivirus (USUV or TBEV) (Ashraf et al., 2015) infections might have had an influence on our results. In one individual of the control group, positive SN titers could be measured at some examinational points, namely on Days 667 and 758, respectively. This increase of SN titers within and some months after the disease season indicate a natural infection (Davidson et al., 2005). Opposite to a previous study, we did not record any clinical manifestation of WNV infection in the non-vaccinated group. In previous studies, only one publication reported natural WNV infection induced neuroinvasive disease in a control group (Chaintoutis et al., 2015). Clinical signs have neither been seen in any of the vaccinated horses during the present study nor in previous experiments (Chaintoutis et al., 2015; Joó et al., 2017; Pearce et al., 2013), and we could also not see an outstanding high titer value which would reflect natural infection and its booster effect on titer values.

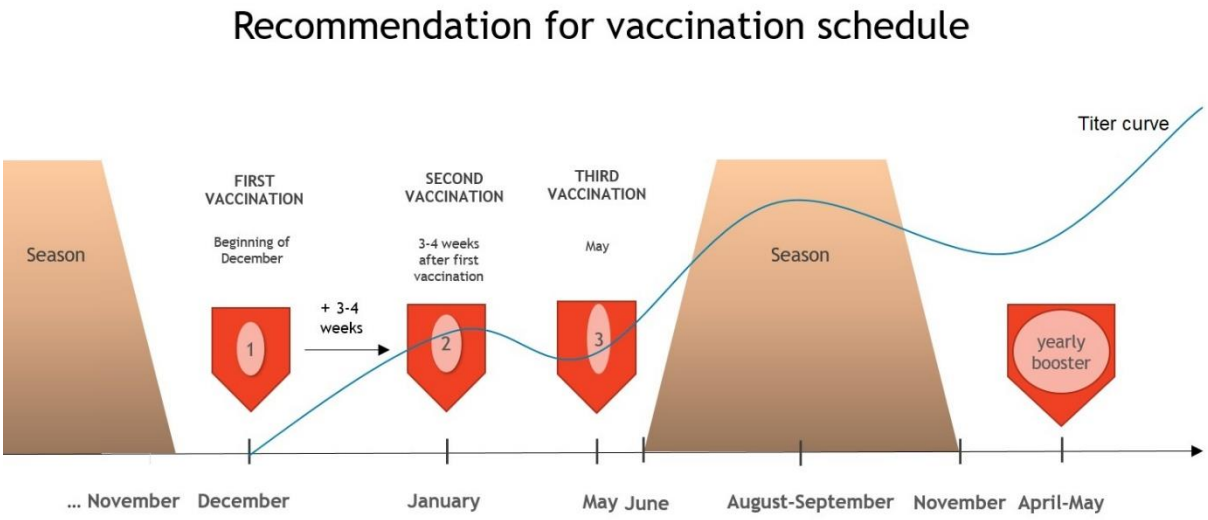


Figure 20. Recommendation for vaccination schedule

Lastly, we should underline the observation that primary vaccination has not induced an anamnestic response in three immunized horses. In the same pattern as Joó et al. (Joó et al., 2017) have previously described, some of the animals appear to be poor responders and in the case of these horses, more frequent vaccination is recommended (Davidson et al., 2005; Joó et al., 2017).

New scientific results

1. Our study is the first comprehensive and descriptive review, containing the clinical data of more than 90 horses infected by lineage 2 West Nile virus, and resulting in West Nile neuroinvasive disease.

2. We describe the temporal and spatial spread dynamics of West Nile virus lineage 2 in Hungary covering a 14 years long data collection period.

3. Our study is the first one to describe in detail the characteristics of the cerebrospinal fluid of horses with West Nile neuroinvasive disease caused by a lineage 2 strain.

4. Our studies describe and evaluate treatment protocols and clinical signs in relation to their prognostic values.

5. Evaluation of the humoral response to the most commonly used inactivated lineage 1 WNV vaccine in Europe and, based on these results, determination of a vaccination protocol in the geographical region of Central Europe.

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Publication and presentation

a) Publications published in foreign scientific journal with an impact factor

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b) Publications published in Hungarian scientific journal with an impact factor

Fehér, O., Szoboszlai, H., Korbacska-Kutasi, O.: Treatment of West Nile Virus caused encephalomyelitis in horses at stable conditions: Case study, Hungarian Veterinary Journal, 2019. (141.), 4. sz., 195-206. p.

b) Conference oral publications

Dr. Fehér Orsolya Eszter, Dr. Malik Péter, Dr. Szögyényi Zsuzsanna, Dr. Halas Máté, Dr. Bakonyi Tamás, Dr. Joó Kinga, Prof. Dr. Szenci Ottó, Dr. Korbacska-Kutasi Orsolya: A Nyugat-nílusi vírus magyarországi előfordulásával kapcsolatos tapasztalatok lovakban, XXVI. Lógyógyászati Kongresszus, Mátraháza, 2018.

Dr. Fehér Orsolya Eszter, Dr. Malik Péter, Dr. Szögyényi Zsuzsanna, Dr. Halas Máté, Dr. Bakonyi Tamás, Dr. Joó Kinga, Prof. Dr. Szenci Ottó, Dr. Korbacska-Kutasi Orsolya: A Nyugat-nílusi vírus magyarországi előfordulásával kapcsolatos tapasztalatok lovakban, MAOK Hajdú-Bihar megyei Éves Továbbképzése, Hajdúszoboszló, 2018.

Dr. Fehér Orsolya: A Nyugat-nílusi vírus magyarországi előfordulásával kapcsolatos tapasztalatok lovakban, MTA ÁLLATORVOS-TUDOMÁNYI BIZOTTSÁGA ÁTE ÁLLATORVOSTUDOMÁNYI DOKTORI ISKOLA AKADÉMIAI BESZÁMOLÓK (2019).

Fehér Orsolya Eszter, Forgách Petra, Marosi András, Malik Péter, Nagy Anna, Takács Mária, Korbacska-Kutasi Orsolya: Nyugat-nílusi vírus és más flavivírusok okozta fertőzések szerológiai vizsgálata, XXVII. Lógyógyászati Kongresszus, Balatonkenese, 2019.

Fehér Orsolya Eszter, Forgách Petra, Marosi András, Malik Péter, Nagy Anna, Takács Mária, Korbacska-Kutasi Orsolya: A Nyugat-nílusi virus és más flavivírusok aktivitása Magyarországon, MTA ÁLLATORVOS-TUDOMÁNYI BIZOTTSÁGA ÁTE ÁLLATORVOSTUDOMÁNYI DOKTORI ISKOLA AKADÉMIAI BESZÁMOLÓK (2020).

Fehér Orsolya Eszter, Piller Pálma, Forgách Petra, Marosi András, Korbacska-Kutasi Orsolya: A Nyugat-nílusi virus okozta megbetegedések során kialakuló immunválasz vizsgálata lovakban, MTA ÁLLATORVOS-TUDOMÁNYI BIZOTTSÁGA ÁTE ÁLLATORVOSTUDOMÁNYI DOKTORI ISKOLA AKADÉMIAI BESZÁMOLÓK (2021).

Supplement material

Clinical examination questionnaire

Official registered number at NÉBIH:
Veterinarian:
Onset of disease:
Sampling date:
Place, county:

General data of equine:

- Age:
- Gender: Female /Male
- Usage: Sport / Hobby / Breeding / No usage
- Stable circumstances: closed stable / field / both
- Vaccination history:
- Previous illnesses:

Clinical signs:

- Hyperthermia
- Anorexia
- Depression
- Behavior change
- Hyperesthesia
- Muscle tremors
- Muscle fasciculation
- Ataxia
- Weakness / Paresis
- Paralysis
- Lamness
- Recumbence
- Colic
- Dysphagia
- Nystagmus
- Cranial nerve paralysis

Treatment:

- NSAID
- Dimethyl- Sulfoxide
- Glucocorticoids
- IV fluid
- Others:

Outcome: survived / died – euthanized

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