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Increased bird of prey mortality in Hungary due to West Nile virus infection

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Abstract West Nile virus (WNV) is a mosquito borne pathogen with a World-wide distribution. The natural hosts of this virus are birds. Although WNV infection often remains subclinical, certain bird and mammal species (particularly horses and humans) may develop febrile illness, and even lethal encephalitis.
An exotic strain of WNV has been detected in 2004 in a goshawk *(Accipiter gentilis)* which succumbed to encephalitis in South-Eastern Hungary. The same strain was detected in 2005 in a goshawk, sparrow-hawks *(Accipiter nisus)* and a sheep, and again in 2007 in goshawks, red-footed falcons *(Falco vespertinus),* domestic geese and in a horse. Up to 2007 all cases were detected in the Great Plain region of the country. In 2008, however, the virus strain suddenly spread all over Hungary. During the epizootic season the virus was detected post mortem in the organs of 12 goshawks and one Harris hawk *(Parabuteo unicinctus)*. The samples were collected in the surroundings of Budapest, and from the Transdanubian regions. The virus also emerged in the eastern regions of Austria, and caused further wild bird mortality.

keywords: West Nile virus, encephalitis, Goshawk, *Accipiter gentilis*, Red-footed falcon, *Falco vespertinus*

Összefoglalás A nyugat-nílusi vírus (West Nile virus, WNV) világszerte elterjedt, szúnyogok által közvetített kórokozó. A vírus természetes gazdái vadmadarak. Bár a WNV fertőzést a gerinces gazdák túlnyomó része tünetmentesen vészeli át, bizonyos madár- és emlősfajokban (különösen lovakban és emberekben) lázas általános tünetekkel járó betegség, de akár halálos kimenetelű agyvelő- és gerincvelő-gyulladás alakulhat ki.
A nyugat-nílusi vírus egzotikus törzsét sikerült kimutatni 2004-ben, Magyarország délkeleti részén, agyvelőgyulladásos tüneteket követően elhullott héja *(Accipiter gentilis)* szerveiből. Ugyanezt a vírustörzset mutattuk ki 2005-ben egy héjából, karvalyokból *(Accipiter nisus)* és egy juhból, majd 2007-ben héjákból, egy kék vércséből *(Falco vespertinus),* háziludakból és egy lóból. A hazai esetek 2007-ig mind a Nagyalföld területén fordultak elő. A vírustörzs 2008-ban elterjedt Magyarország területének jelentős részén. A járványidőszakban kimutattuk a vírust 12 héja és egy Harris ölyv *(Parabuteo unicinctus)* szerveiből. Az elhullott madarak Budapest környékéről és a Dunántúlról származtak. A vírustörzs felbukkant Ausztria keleti részén is, ahol további vadmadár-elhullásokat okozott.

kulcsszavak: nyugat-nílusi láz, agyhártyagyulladás, héja, *Accipiter gentilis,* kék vércse, *Falco vespertinus*

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West Nile virus (WNV) a member of the family Flaviviridae, genus *Flavivirus*, is the causative agent of West Nile fever (WNF), a disease characterized by fever, general flu-like signs, and encephalitis in different vertebrate hosts (Heinz et al. 2000).

Under natural conditions WNV was found to infect and circulate in wild bird populations, transmitted by haematophagous arthropods, mainly mosquitoes. Certain bird species contribute to the maintenance of virus circulation through acute infections with

high-titre viraemia (amplifying hosts), but the infection in these birds usually remains subclinical. Infections of other hosts, such as other bird species and mammals (predominantly horses and humans), may lead to the development of clinical signs, and even fatal central nervous system (CNS) disease. Mosquitoes play a central role in the infection of incidental hosts (mammals), acting as bridge vectors between them and the reservoir birds.

Due to its global distribution and the damages it caused in horse and bird populations, as well as the zoonotic risk it poses to humans, over the last decade emphasis has been placed on the research into the characteristics of WNV (Hayes et al. 2005a, b).

Etiology

West Nile virus is an enveloped virus with icosahedral capsid symmetry and single-stranded, positive-sense RNA genome (Rice et al. 1986). Hemaglutination inhibition and cross neutralization data have demonstrated that WNV is a member of the Japanese encephalitis vitus (JEV) serocomplex. Other members of the JEV group flaviviruses are Cacipacore virus (CPCV), Koutango virus (KOUV), St. Louis encephalitis virus (SLEV), Murray Valley encephalitis virus (MVEV), Alfuy virus (ALFV), Usutu virus (USUV), and Yaounde virus (YAOV) (Heinz et al. 2000).

WNV has several strains that show relatively wide genetic diversity. The studies conducted on their phylogenetic relatedness revealed that WNV strains could be grouped into two main lineages (Berhet et al. 1997, Lanciotti et al. 2002, Charrel et al. 2003). Lineage 1 contains WNV strains from different regions and is divided into three clades. Clade "a" contains strains from Europe, Africa, Near East and America. Clade "b" represents Australian (Kunjin) strains while Clade "c" comprises strains isolated in India. Lineage 2 viruses were found only in the sub-Saharan regions of Africa and Madagascar before. Recent isolation of new genotypes in Central Europe, Russia and India, suggests the existence of further genetic lineages of WNV (Bakonyi et al. 2005, Bondre 2007).

Distribution

WNV was first identified in the West Nile district of Uganda in 1937. Blood collected from an African woman suffering from mild fever revealed a virus that was later called West Nile virus (Smithburn et al. 1940). Although antibodies against WNV are commonly found in Africa, association with human disease was not apparent at first. The same virus was later (1951) isolated in Egypt from healthy children, wild birds, mosquitoes and from the brain of a horse with encephalitis (Melnick et al. 1951).

Subsequently West Nile virus has been identified as the causative agent of endemics and epidemics of viral encephalitis in Europe, Africa, Asia, and has resulted in an epidemic outbreak in the United States (US) in 1999 (Hubálek & Halouzka 1999). The epidemic in the US occurred during late summer, in the north eastern states, causing an outbreak of human encephalitis as well as concurrent widespread mortality in crows and exotic birds of a zoological park (Anderson et al. 1999). WNV has since spread west across the continent, north to Canadian provinces and south to Mexico (Blitvich et al. 2003). After its

emergence WNV caused >17 000 human infections and >670 deaths (CDC, 2005) within six years.

The spread of West Nile virus across the Old World has been attributed to migratory birds belonging to the Palearctic-African migration system or wintering in the Mediterranean area. These species presumably play a central role and are responsible for periodic introductions of African or Near Eastern WNV strains into Europe (Hubálek 2000). The strain, which emerged in the USA most likely originated from Israel, however the route of its introduction remained unclear (Giladi et al. 2001).

Ecology and epizootiology

The WNV natural cycle involves the main vertebrate hosts (avian species) and arthropod vectors, which feed on the hosts. Vectors responsible for the spread of WNV are mosquitoes of various genera, with *Culex* being the predominant one. When a female mosquito takes a blood meal from an infected host (bird) they only become infective after 10-14 days of incubation. After this time they can transmit WNV through a second blood meal. Most birds involved in this enzootic cycle do not develop disease, and hence act as reservoirs. The more susceptible species, avian and mammalian, are typically infected when a "bridge-vector" feeds on an infected host and then, after an extrinsic incubation time, on the susceptible species. This is thought to be the method of transmission of WNV to humans, horses and other species (Taylor et al. 1956).

Host to host horizontal transmission has been found possible when bird secretions (oral or cloacal) contaminate water and food (Banet-Noach et al. 2003). Research has also shown that the virus may be passed to raptors by eating infected birds (Garmendia et al. 2000). Ticks have been found infected with the virus in Asia and Africa, but there are no verified reports of ticks spreading the virus, therefore their role in transmission has not been determined (Hayes 1989).

Pathogenesis

When WNV is inoculated into the skin via a mosquito bite, a local replication of the virus starts, and later the virus spreads to the regional lymph nodes. The virus is carried further via the lymphatic system to the thoracic duct to then enter the systemic circulation. The level of the viraemia is affected by the rate of clearance of macrophages and it ends when humoral antibodies appear, one week post infection.

Viral penetration into the CNS appears to follow the stimulation of toll-like receptors and increase in levels of tumor necrosis factor-α, which increases the permeability of the blood-brain barrier. WNV directly infects neurons in the grey matter and deep nuclei of the CNS. Simultaneous destruction of bystander nerve cells may contribute to signs of paralysis. It is believed that immune mediated tissue damage also contributes to pathological consequences. The neuroinvasive character of WNV varies between different isolates, regardless which genetic lineage they belong to.

Clinical signs and pathological lesions

Most WNV infections are inapparent and subclinical, however approximately 20% of the infections result in signs ranging from

mild fever to fatal encephalitis in humans, horses or birds (Banet-Noach et al. 2003).

Clinical signs of WNF in humans are usually mild and include fever, headache, body aches and, in some cases, skin rash and swollen lymph glands (Watson et al. 2004). Severe infections include high fever, neck stiffness, muscle weakness, convulsions and paralysis. Death rates associated with severe infection range from 3% to 15% and are highest among the elderly. In horses the most common sign is weakness in the hindquarters, indicated by a widened stance, stumbling, leaning, and toe dragging. In extreme cases, paralysis develops. Sometimes other signs, such as fever, depression and fearfulness, can follow. Approximately 33% of cases of WNV encephalitis in horses proved fatal during the 2001 outbreak in the United States (Porter et al. 2003).

In several wild bird species the disease is not apparent, making them good reservoirs that mobilise the virus along their migration paths. In contrast, most North American corvids infected with WNV die within 3 weeks of infection (Komar et al. 2003). Clinical signs are general and may include incoordinated walking, weakness, lethargy, tremors, and abnormal head posture. In North America, wild birds infected with WNV are most often found dead; therefore, descriptions of clinical signs in these cases are not readily available. Domestic birds such as chicken do not seem to develop the disease; however ducks and pigeons develop similar signs to those observed in wild birds (Kramer & Bernard 2001). Clinical signs associated with WNV infection in dogs, cats, domestic rabbits and other small mammals have not been well described. It appears that, although they may be infected, many members of these latter species rarely develop clinical signs of disease. However if clinical signs do develop, they include fever, listlessness, stumbling, lack of coordination, ataxia, partial paralysis and death (Austgen et al. 2004).

WNV shows no pathognomic lesions. Observed changes in humans and experimental animals with WNV encephalitis include neuronal and glial damage caused directly by viral replication and characterized by central chromatolysis, cytoplasmic eosinophilia, cell shrinkage, and neuronophagia; inflammation, including perivascular infiltration of small lymphocytes, plasma cells, and macrophages; cellular nodule formation composed of activated microglia and mononuclear cells; and cerebral interstitial oedema (Sampson et al. 2000). Infection of neurons is characterized by marked proliferation and hypertrophy of rough endoplasmic reticulum (RER), accumulation of vesicular structures derived from the RER and containing virus particles, and progressive degeneration of the RER and Golgi apparatus. It is suggested that neuron dysfunction is responsible for fatality in the host, as apposed to neuronal destruction. Residual changes and disturbances have been noted to persist after recovery from acute encephalitis. In experimental animals, changes in behaviour and learning ability have been documented (Austgen et al. 2004).

In birds, necropsy usually does not show pathological signs indicative of WNV infection, but histologically the signs of encephalitis are visible (Komar et al. 2003). In horses, no gross pathologic lesions have been detected. Histologically, animals exhibit slight to moderate nonsuppurative encephalomyelitis, primarily in the spinal cord and lower brainstem, in both grey and white matter. The most severe lesions are usually in the thoracic and lumbar spinal cord (Castillo-Olivares & Wood 2004).

Diagnosis

Subclinical WNV infections are rarely detected with direct diagnostic methods. In the acute (febrile) phase of infection the virus can be detected in the serum, in the peripheral blood mononuclear cells, and in some cases also in oral swabs and faecal samples. Viraemia and virus shedding is usually short-termed. If CNS signs develop, the virus is often present in the cerebrospinal fluid, and in the tissues of the brain and spinal chord. Tissue samples of the spleen, liver, kidney, lung, trachea, heart, small intestine, pancreas, and thyroid gland are also potential sites of direct virus detection. WNV may be isolated by intracerebral inoculation of suckling mice, inoculation of the allantoic or amniotic sac of embryonated eggs, or infection of susceptible *in vitro* cell cultures (e.g. primary goose embryo fibroblast tissue, Vero cells, etc.). Because WNV is also a human pathogen, isolation attempts must be performed under biosafety level 3 conditions at least.

The reverse transcription polymerase chain reaction (RT-PCR) is the most frequently used molecular method to detect the viral RNA in diagnostic samples. Besides its high sensitivity and specificity, it also allows subsequent genetic characterization of the detected virus. Virus antigen can be detected in tissue sections using specific antibodies in immunohistochemistry and direct immunofluorence assays (Erdélyi et al. 2007).

The indirect diagnosis of WNV infections is based on the detection of specific anti-viral antibodies. The various serological methods may differ in specificity and in sensitivity. Since flaviviruses share common surface antigens, non-specific cross-reactions may compromise serological diagnosis. Certain serological methods, for example ELISA, haemagglutination-inhibition, or indirect immunofluorescence are more suitable for initial screening of serum samples for flaviviral antibodies, while the specificity of the antibodies in positive samples should subsequently be validated by plaque reduction neutralisations tests (PRNTs).

Cases that manifest in clinical signs, are most often of the neurological type, and must therefore be differentiated from other diseases causing neurological disorders.

In birds WNV must be differentiated from Newcastle disease, Avian encephalomyelitis, Avian influenza, Marek's disease, Eastern encephalitis and non viral causes of neurological disorders as avian encephalomalacia (vitamin E deficiency), vitamin B1 or B2 deficiency, and encephalitis caused by bacteria, fungi (i.e. aspergillosis), or mycoplasmas.

In horses, differential diagnostics includes Equine protozoal myeloencephalitis, Cervical vertebral myelopathy, Equine herpesvirus 1 infection, Equine degenerative myelopathy, Western-, Eastern- and Venezuelan equine encephalitis, Japanese encephalitis, Borna disease or botulism. Considering the ascending paralysis, mentation changes, and hyperesthesia in some cases, all horses that die or are euthanized should be sent for rabies diagnostics.

Management and control

In endemic season the transmission cycle of WNV is influenced by the presence and abundance of amplifying bird hosts and competent mosquito vectors. Reducing the number of vectors in urban and suburban areas by controlling and limiting mosquito breeding sites (like ditch water, small recep-

tacles containing stagnant water and small water bodies), and prevention of mosquito bites by the use of repellents, netting, and other methods are reasonable approaches for the prevention of human and domestic animal (i.e. horse) infections. In the nature, however, the application of pesticides raises serious concerns, and the efficacy of this approach is rather weak. Attempts at the control of WNV infections in wild animal populations have not been reported so far. Passive surveillance schemes of human and horse encephalitis cases, complemented by passive surveillance of bird mortality and sentinel birds in endemic areas are useful tools for the identification of WNV activity; therefore they are recommended by the EU Scientific Committee on Veterinary Measures Relating to Public Health.

Active immunization with inactivated or recombinant WNV vaccines designed to be used in horses has been applied in captive individuals belonging to susceptible bird species (e.g. birds of prey). The efficacy of vaccination seems to vary on a wide scale, and it is apparently influenced by the type and make of the vaccine as well as the specific host species.

Treatment protocols for clinical WNV cases are limited. Supportive therapy and supplementary feeding have been found to be essential to maximize the chances of recovery of individual patients from WNV infection. Captive, usually older, goshawks which survive the acute phase of the infection and maintain good body condition are found to recover from the infection, although chronic lesions resulting in impaired vision or minor neurological disorders may remain in some cases.

West nile virus in Hungary

The presence of WNV in Hungary was already recognized on the basis of serological studies of humans more than forty years ago (Koller et al. 1969), and the virus was isolated from rodents (bank vole, *Clethrionomys glareolus,* 1972, and yellow-necked mouse, *Apodemus flavicollis,* 1976) (Molnár 1982). However, clinical disease was never recognised until 2003, when an outbreak of enzootic encephalitis emerged in a Hungarian goose flock resulting in a 14% mortality rate of 6 weeks-old geese *(Anser anser domesticus)*. Based on histopathological lesions, serological investigations and nucleic acid detection by RT-PCR, WNV was diagnosed as the causative agent of the disease (Glávits et al. 2005). At the same time, an endemic WNV outbreak was also observed in humans in Hungary, which involved 14 reported cases of mild encephalitis and meningitis (Ferenczi et al. 2005).

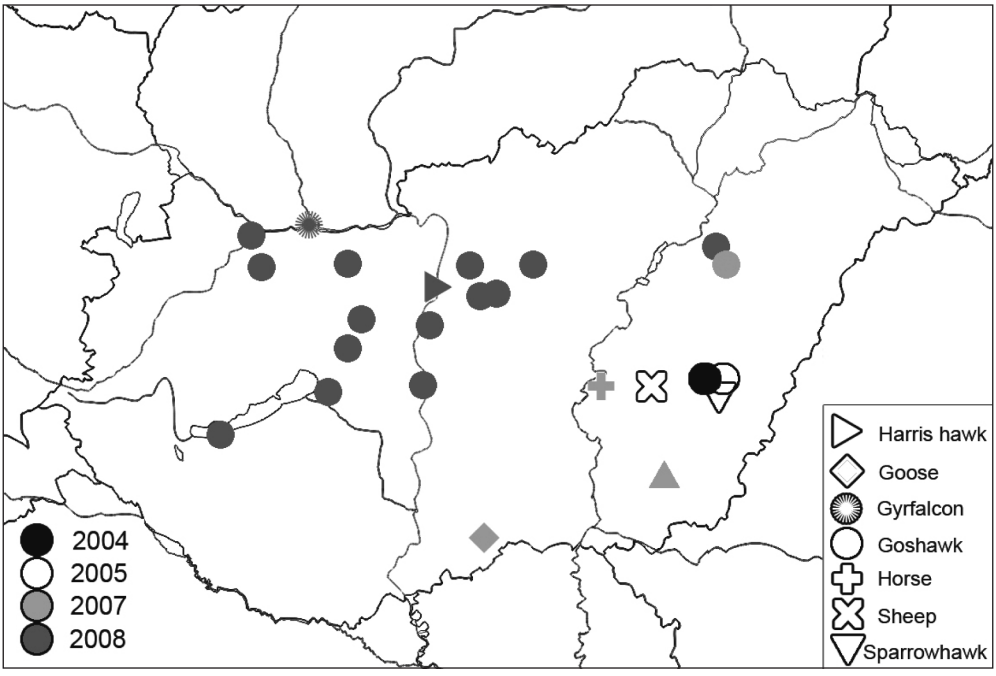
One year later, in September 2004, a goshawk *(Accipiter gentilis)* showing CNS signs died in a rehabilitation facility of the Körös-Maros National Park at the south-eastern part of Hungary. The observed clinical signs were sudden in onset and comprised ataxia, head tremors and seizures in the terminal phase. On post-mortem, macroscopic lesions were found to be non-specific, showing only the congestion of internal organs. Examination of hematoxylin-eosin stained histological sections of the central nervous system revealed multi-focal, lymphocytic panencephalitis with marked gliosis, and neuronal degeneration comprising chromatolysis, necrosis and neuronocytophagia. Additionally, demyelinisation of the cerebellar white matter and lymphocytic meningitis were detected, and mild, focal mononuclear infiltration was present in peripheral nerves of the

sciatic plexus. Spleen displayed multi-focal proliferation of reticulocytes and lymphoid depletion. Multi-focal lympho-histiocytic myocarditis was detected in the heart. Additional findings, like multi-focal interstitial lympho-histiocytic nephritis, lung congestion, and mild lymphocytic enteritis and proventriculitis were also detected in the bird (Erdélyi et al. 2007). Using immunohistochemistry and RT-PCR, both WNV antigen and nucleic acid were detected in the organs of the bird (Erdélyi et al. 2007).

The virus isolated in 2003 was identified as a lineage 1, while the virus isolated in 2004 as a lineage 2 West Nile virus (Bakonyi et al. 2006). The latter strain has thereafter established itself in Hungary. In 2005, sporadic WNV cases were detected again in birds of prey (goshawk, sparrowhawk *[Accipiter nisus]*) and the virus was also isolated from the brain of a sheep, which died of encephalitis in 2005 (Erdélyi et al. 2007, Kecskeméti et al. 2007).

In 2007 WNV was detected in goshawks in the territory of the Hortobágy National Park, in domestic goose farm in southern Hungary, and in a red-footed falcon *(Falco vespertinus)* colony in the Körös-Maros National park. Birds of prey were found dead, lymphocytic encephalomyelitis was observed, and WNV antigens and nucleic acid were demonstrated in the organ samples collected from the birds. The geese have shown CNS signs and lameness. Besides encephalitis, muscular dystrophy was found in the birds. The first WNV-associated encephalomyelitis in horse in Hungary was also diagnosed in 2007 (Kutasi et al. 2011). While the cases in 2004-2005 were detected relatively close to each other (~ 30 km in a straight line), the cases in 2007 have shown wider geographic distributions (150–200

Figure 1 Temporal and geographic distribution of WNV cases diagnosed in wild and domestic animals in Hungary between 2004 and 2008.



km from each other), but were confined to the Great Plain region of the country.

Suddenly, a significant geographic expansion of the virus strain was observed in the summer and autumn of 2008. WNV was detected in 17 Goshawks, in 2 Harris hawks *(Parabuteo unicinctus),* in 3 Gyrfalcons *(Falco rusticolus),* in a Red-footed falcon, in a European roller *(Coracias garrulus),* and in a Barn owl *(Tyto alba)*. Goshawks were collected in the central and Transdanubian regions of Hungary. Many of the birds were kept by falconers, and have shown CNS signs prior to death. The Red-footed Falcon, the European roller, and the Barn owl were found near the Red-footed Falcon colony, where WNV activity was also diagnosed in 2007. Simultaneously, horse (3 direct and 14 indirect diagnoses) and 14 human cases were also diagnosed in the enzootic season (between August and October) *(Figure 1.)*. The same virus strain also emerged in birds of prey in the eastern region of Austria (Wodak et al. 2011). Since 2004, in all diagnosed Hungarian WNV cases, the lineage 2 strain was demonstrated in the samples. It indicates that the virus established a successful infection cycle, became resident in the country, and was able to cause neurological disease.

Conclusions

Outbreaks of West Nile fever in the Mediterranean and Eastern Europe have been well documented over the past century. The source of these WNV outbreaks has been contributed to introductions from endemic areas in Africa and subsequent amplification of the virus through limited local circulation. Migratory birds have been implicated in the geographic spread and repeated introduction of WNV into non-endemic areas. The European activity of lineage 1 WNV strains has been well recognised but until recently lineage 2 strains were thought to have a limited distribution in Africa and Madagascar. The unexpected emergence of lineage 2 WNV in the Carpathian basin (Hungary and Austria) and the indications of its endemic establishment in the area resemble the North American introduction of WNV on a smaller scale. It is not known which factors are crucial for the establishment of WNV in Europe. The parallels drawn with the North American findings and research results should be verified in the European context, as there seem to be marked differences in several aspects of WNV epidemiology between the two continents. The lack of bird mortality indicating WNV presence is one such peculiarity. Our findings suggest that birds of prey, especially goshawks, are good indicators of WNV presence. Mass mortality due to WNV may not be expected in Europe, so a much subtler, targeted surveillance is needed to detect virus circulation in endemic or high risk areas. In the light of the situation in Hungary and other endemic areas (especially in Italy and in Romania) a further geographic spread of WNV may be expected. The increased activity of WNV automatically results in a heightened risk of infection for humans. As the circulating lineage 2 strain is also highly neurotropic the higher risk of WNV encephalitis and the potential risk associated with blood transfusion should not be neglected. The detection of WNV encephalitis in horses and sheep indicates that veterinary aspects of WNV will also have to be reckoned with in the future. The spread of WNV may potentially present us with conservation issues. Although the majority of presently affected birds of prey are not strictly endangered species, their ecology

suggests that populations of other sedentary, isolated, naive bird species may eventually be considered at risk if contact with WNV would eventually get established.

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