Equine Encephalomyelitis Outbreak Caused by a Genetic Lineage 2 West Nile Virus in Hungary

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Background: The spread of lineage 2 West Nile virus (WNV) from sub-Saharan regions to Europe and the unpredictable change in pathogenicity indicate a potential public and veterinary health threat and requires scientific awareness.

Objectives: To describe the results of clinical and virological investigations of the 1st outbreak of a genetic lineage 2 WNV encephalomyelitis in horses.

Animals: Seventeen horses with neurologic signs.

Methods: Information regarding signalment, clinical signs, and outcome was obtained for each animal. Serology was performed in 15 cases, clinicopathological examination in 7 cases, and cerebrospinal fluid was collected from 2 horses. Histopathology was carried out in 4 horses, 2 of which were assessed for the presence of WNV in their nervous system.

Results: WNV neutralizing antibody titers were between 10 and 270 (median, 90) and the results of other serological assays were in agreement with those of the plaque reduction neutralization test. Common signs included ataxia, weakness, asymmetric gait, muscle tremors, hypersensitivity, cranial nerve deficits, and recumbency. Twelve animals survived. Amplicons derived from the infection-positive specimens allowed molecular characterization of the viral strain.

Conclusions and Clinical Importance: From our results, we conclude that this outbreak was caused by a lineage 2 WNV strain, even though such strains often are considered nonpathogenic. Neurological signs and survival rates were similar to those reported for lineage 1 virus infections. The disease occurrence was not geographically limited as had been the typical case during European outbreaks; this report describes a substantial northwestern spread of the pathogen.

Key words: Horse; Nervous system; West Nile virus.

Phylogenetic studies have identified 2 main lineages of West Nile virus (WNV) strains. Strains from lineage 1 are present in Africa, India, and Australia and are responsible for outbreaks in Europe, in the Mediterranean Basin, and in North America, whereas lineage 2 strains have been reported only in sub-Saharan Africa and Madagascar.[1–3] The higher morbidity and mortality rates because of WNV lineage 1 strains relative to lineage 2 strains led to the supposition that lineage 1 strains are highly pathogenic whereas lineage 2 strains endemic to Africa are less virulent.[4] However, it was subsequently demonstrated that lineage 2 strains also may cause severe disease.[1] Furthermore, experiments using mice showed marked differences in the neuroinvasive phenotype that did not correlate with the lineages, suggesting that highly and less neuroinvasive phenotypes exist in both lineages.[1] In Hungary, the WNV strain that causes lethal encepha-

Abbreviations:

BLAST	basic local alignment search tool
CSF	cerebrospinal fluid
ELISA	enzyme-linked immunosorbent assays
FAM	6-carboxy-fluorescein
HIT	hemagglutination inhibition test
IFAT	indirect fluorescent antibody test
MEGA	molecular evolutionary genetic analysis
PBMC	peripherial blood mononuclear cell
PRNT$_{90}$	plaque reduction neutralization test, endpoint 90% neutralization level
RT-PCR	reverse transcription polymerase chain reaction)
TAMRA	6-carboxytetramethyl-rhodamine;
WBC	white blood cell
WNV	West Nile virus

litis emerged in geese in 2003 but there were no recognized equine clinical cases or positive postmortem diagnoses established until the autumn of 2007. In 2004 and 2005, lethal WNV encephalitis was recorded in birds of prey and in a single sheep.[5,6] A lineage 1 strain was detected in 2003 from geese, and this strain exhibited the closest genetic relationship to the strains isolated in Israel in 1998 and to the strain that emerged in the United States in 1999.[7] The cases from 2004 and 2005, and an equine case from 2007, were caused by a strain belonging to lineage 2 that was previously isolated only in sub-Saharan Africa.[5,8] In 2008, WNV was found to be responsible for neurological conditions in horses in Italy, in addition to Hungary.[9]

The aim of the present study is to describe the results of clinical and virological investigations of the 1st outbreak of a genetic lineage 2 WNV encephalomyelitis in horses in Hungary.

Materials and Methods

Clinical and virological data were collected from 17 horses showing neurological symptoms between August and October in 2008. None of the horses were vaccinated for WNV.

Routine physical and specific neurological examinations were undertaken for all horses. Venous blood was withdrawn from 7 horses for CBC with an automated hematology analyzer,[a] and serum biochemistry including glucose, protein, and various enzymes with a spectrophotometer.[b] Cerebrospinal fluid (CSF) was collected from the atlantooccipital space after induction of general anesthesia with xylazine[c] (1.1 mg/kg), diazepam[d] (0.1 mg/kg), and ketamine[e] (2.2 mg/kg) in 2 cases showing signs of encephalitis. The appearance of the CSF and cytological features, protein concentration, and specific IgG concentration in the CSF were determined.

Complete necropsy was carried out in 4 horses, and representative samples of the central nervous system were fixed in 10% neutral buffered formalin and processed routinely for histopathology. Histological sections were stained with hematoxylin-eosin.

Fifteen horses were serologically tested, and WNV infection was proven by histopathology and polymerase chain reaction (PCR) examinations in 2 other cases. Antibody responses such as IgM, IgG, or neutralizing responses were evaluated according to the Word Organization for Animal Health guidelines by enzyme-linked immunosorbent assays (ELISA) or the plaque reduction neutralization (PRNT$_{90}$) test. Sera also were tested with the hemagglutination inhibition test (HIT) and indirect fluorescent antibody tests (IFAT). IFAT was used for the detection of WNV IgG and IgM antibodies on in-house slides. Vero cells[f] were infected with a lineage 1 WNV strain that was isolated in Hungary.[10] This strain's nucleotide sequence is similar to the Eg 101 prototype strain (isolated in Egypt in 1951, unpublished data). The infected cells were harvested on the 3rd day after infection, trypsinized, and spotted on slides. The IgG content of sera were tested first at a 1 : 10 dilution of serum and at subsequent 2-fold dilutions, and then incubated in a wet chamber for 1 hour 37°C, washed (3 times for 5–10 minutes and incubated for 30 minutes with fluorescein-conjugated anti-horse IgG[g] in a wet chamber at 37°C. Sera tested for IgM were chromatographed by ion-exchange chromatography[11] and examined at final serum dilutions of 1 : 10 and 1 : 20 and incubated for 3 hours in a wet chamber at 37°C. After washing (3 times for 5–10 minutes), the samples were incubated for 30 minutes with anti-horse IgM conjugate[g] in a wet chamber at 37°C. CSF samples were tested undiluted for IgG and IgM. Positive cases were examined for other flavivirus infections (tick-borne encephalitis and Usutu viruses) by performing parallel tests. Paired sera were always titrated side-by-side on the same antigen.

The HIT using an in-house hemagglutinating antigen was performed by the classical method described previously.[12]

In an attempt to characterize the virus, isolation, nested reverse transcriptase (RT)-PCR, real-time RT-PCR, and sequencing techniques were used. Peripheral blood samples from 10 symptomatic horses were collected in 4 mL collection tubes containing tripotassium ethylenediaminetetraacetic acid (K_3EDTA).[h] Peripheral blood mononuclear cells (PBMCs) were purified from the buffy coat fractions of the blood samples with Buffer L, which is an erythrocyte lysis buffer.[i] Nervous system tissues from the lumbosacral region of a 6-month-old foal and from the brainstem of an 8-year-old mare were examined. Brain and spinal cord samples were frozen and stored at −80°C before virological investigations. Nervous system tissue samples first were homogenized in ceramic mortars with sterile quartz sand, and the homogenates were suspended in Minimal Essential Medium.[j] Organ samples were centrifuged at $1,500 \times g$ for 10 minutes, and viral RNA was extracted from 140 µL of the supernatants with the QIAamp Viral RNA Mini Kit.[i] Viral RNA also was purified from the PBMCs with the same kit according to the specific protocol provided by the manufacturer. The WNV nucleic acid was amplified by RT-PCR as described pre-

viously.[8] Samples first were tested with a universal Japanese encephalitis virus specific serocomplex,[13] as well as with WNV lineage 1 and lineage 2 specific oligonucleotide primer pairs. The samples additionally were tested with a TaqMan technology-based real-time RT-PCR assay. The target region of the assay is the NS3 protein coding region of the WNV lineage 2 strain's genome (genomic primer: WNV 5009f, 5′-GAACGTCAGGTTCCCCCATT-3′; complementary primer: WNV 5103r, 5′-GGCGCTTATGTATGAACCATTA GG-3′; TaqMan probe: WNV 5050p, FAM-5′-ATTGGATTGTATG GGAACGGCGTCATC-3′-TAMRA). The SuperScript III Platinum One-Step Quantitative RT-PCR System Kit[k] was used for the amplifications according to the manufacturer's instructions; primers and probe were applied at a 0.2 µM concentration. Reaction mixtures (25 µL) contained 2.5 µL template RNA, and all samples were tested in duplicate. Amplifications were performed in an 7,300 Real-Time PCR System[l] with 96-well plates. The thermal profile of the reactions were as follows: 48°C for 15 minutes (RT), 95°C for 2 minutes (Taq activation), and 45 cycles at 95°C for 15 seconds and 60°C for 30 seconds (amplification).

Nucleotide sequences of the amplification products were determined by the fluorescence-based direct sequencing method. Sequences were identified by a BLAST search (http://blast.ncbi.nlm.nih.gov/ Blast.cgi) and were subjected to phylogenetic analysis by the neighbor-joining statistical algorithm. Details of the methods have been described earlier.[8]

Specimens that were diagnosed as WNV positive by RT-PCR were subjected to virus isolation by inoculation into suckling mouse brain and in Vero cell lines, as described previously.[5] Isolates were identified by partial nucleic acid sequence determination after RT-PCR on the 2nd or 3rd passages.

Results

The mean age of the affected horses was 8.0 (SD, 3.8) years, ranging from a 6-month-old Thoroughbred Colt to a 16-year-old Standardbred mare. Affected horses included 8 mares, 6 geldings, and 3 stallions, and originated from different parts of the country with a distribution that was reflective of the typical hospital population. Most of the horses were Standardbreds (10/17), but Thoroughbreds (3/17), Shetland Ponies (3/17), and 1 Friesian also were diagnosed with the disease.

The 1st case occurred on August 26 and the last one on October 10, 2008 (Fig 1). Only 3 horses had low-grade fever at the onset of the disease. The most common signs were ataxia (in 13/17 of cases) and weakness (in 13/17 of cases). Weakness and ataxia were more pronounced in the forelimbs in 4 horses and an asymmetric gait was observed in another 4 horses. Fasciculation of triceps, quadriceps, head, and neck muscles (7/17), hypersensitivity (5/17), and muscle rigidity (5/17) also were observed. Two horses showed behavioral changes and 4 had signs of cranial nerve deficits such as dysphagia, salivation, and unilateral facial paralysis. There was progression of paresis to tetraplegia and recumbency in 7 horses, 5 of which were euthanized or died. Nine animals completely recovered within 2 months, whereas 3 horses continued to show signs of facial paralysis, hindlimb ataxia, or both at 6 months after the acute phase. Serological investigations indicated positive antibody responses in all tested horses. Serology was considered confirmatory for acute WNV infection when a 4-fold increase was found for IgG in 2 serum samples of 1 animal,
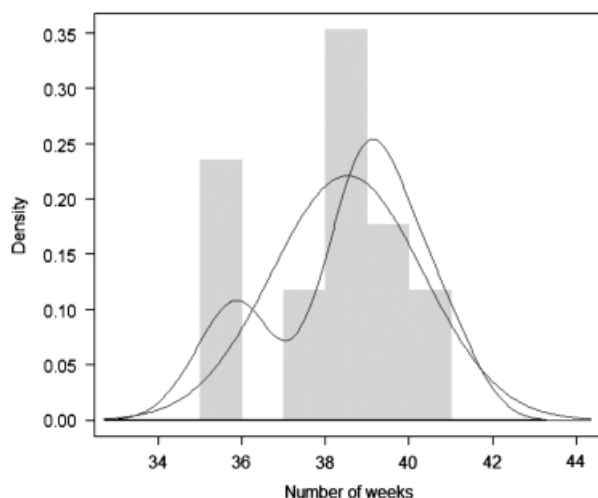
Fig 1. Histogram of the number of cases with estimated normal curve and estimated general density curve.

IgM positivity in at least one of the methods used or both. Neutralizing antibody titers ranged between 10 and 270 (median, 90) at 2–35 days after the onset of clinical signs. Results of other serological assays were in agreement with the $PRNT_{90}$ test. Hematology was usually negative but showed increased white blood cell (WBC) count and neutrophilia in 3 fatal cases. Metabolic profiles were within reference values, except for increased plasma protein concentration (>8.9 g/dL) in some horses (n = 2) and high creatine kinase activity in recumbent animals ($>3,233$ IU/L). The 2 CSF samples were clear and colorless but revealed pleocytosis with mainly neutrophils and a low number of small lymphocytes on cytology, increased protein level (>210 mg/dL), and one showed a weak antibody response. Gross pathology was negative in each case. The neuropathological pattern was characterized by lymphocytic-plasmacytic perivascular infiltration and gliosis and some focal neutrophil-granulocyte infiltration with neuronal degeneration (Fig 2). Alterations mostly were found in the brainstem, medulla,
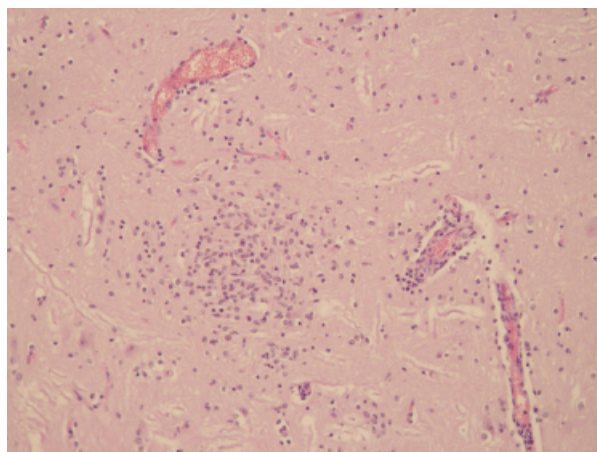

Fig 2. Focal glial proliferation and nonsuppurative perivasculitis and encephalitis because of West Nile virus lineage 2 infection; horse brain, hematoxylin-eosin, 200 ×.

and gray matter of the spinal cord, with the most severe lesions in the cervico-thoracic and in lumbar regions. Two horses were assessed for the presence of WNV in their nervous system (brainstem and lumbar region) by RT-PCR; both specimens were positive and allowed for molecular characterization of the viral strain. PCR testing on leukocytes resulted in 1 of 10 horses testing positive for WNV. The phylogenetic tree that was drawn after multiple alignment with diverse lineage 1 and 2 strains clearly shows that the currently circulating WNV in Hungary belongs to lineage 2 (Fig 3).

Discussion

The Hungarian equine WNV outbreak reported herein was the first to be caused by a lineage 2 sub-Saharan strain in Europe. The pathogenicity of this lineage 2 strain resembled that of lineage 1 strains, and its sudden spread was unpredictable.

The survival rate in our study was approximately 70%, which is similar to the data previously reported for lineage 1 outbreaks.[9,14] In our study, 25% of horses had sequelae at approximately 6 months after the diagnosis. In a study by Wilson et al,[15] 40% of the horses in an outbreak in the United States experienced permanent nervous system abnormalities. In contrast, full recoveries were reported in a small outbreak in Italy in 1998, in which 8 survivors had no permanent sequelae.[16] Horse owners' perception of what constituted a full recovery was not reliable. Only 1 horse owner reported gait abnormality, but sequelae were detected upon veterinary re-evaluations in the other 2 cases. Weakness, ataxia, and paralysis are typical features of nervous system involvement in WNV infections, but these usually develop primarily in the hindquarters.[14,15,17,18] In four of our cases, the signs were more prominent in the forelimbs at the onset of the disease. Fever and cranial nerve abnormalities have been observed in some previous outbreaks.[16,17] Forelimb ataxia, asymmetric gait and head posture, fever, and hyperexcitability also were described in a South African retrospective study on lineage 2 equine infections.[19] Factors other than the strain alone may account for the clinical manifestation of disease. Individual factors of receptivity also are thought to be of major importance for the evolution and clinical expression of WNV infection. Indeed, several authors have demonstrated that variations in certain loci of the host's genome influenced susceptibility and clinical presentation.[20] Furthermore, the presence of antibodies against other flaviviruses is thought to play a role in determining the clinical presentation in certain areas.[21]

In the report by Cantile,[20] detailed laboratory results were lacking in many case descriptions, but hematology was found to be negative in most horses. We found pronounced increases in WBC counts with neutrophilia in 3 cases. Regarding human cases with central nervous system involvement, increased WBC counts were reported in approximately 40% of patients in an Israeli outbreak, and mild increases in mean WBC count and prolonged lymphocytopenia have been reported in several US cases.[22] Neutrophil predominance in CSF was a unique finding among clinical data in horses, but it is
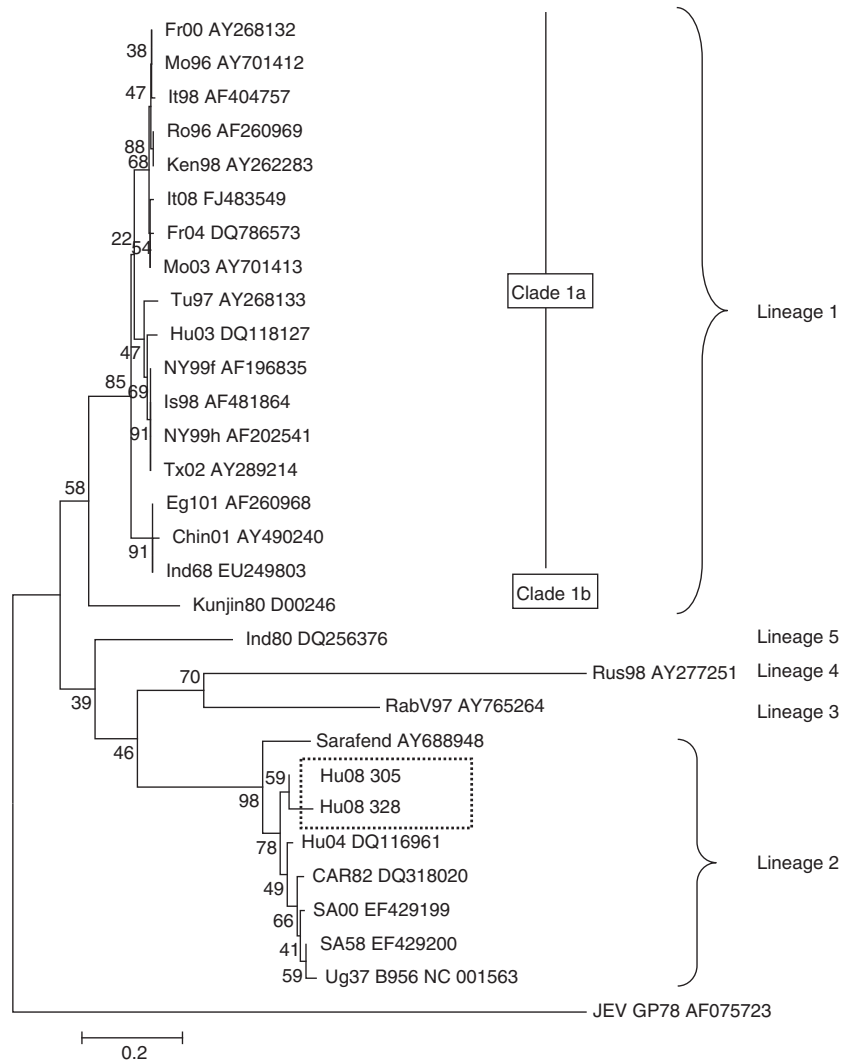
Fig 3. Phylogenetic tree of West Nile virus strains based on a 282nt fragment of the Envelope gene. The tree was constructed with the program MEGA (Molecular Evolutionary Genetic Analysis) by neighbor-joining. Bootstrap confidence level (1,000 replicates) and a confidence probability value based on the standard error test were calculated by MEGA. WNV strains are named according to the following rules: a set of letters corresponding to the place where the strain was isolated (Fr, France; Mo, Morocco; It, Italy; Ro, Romania; Ken, Kenya; Tu, Tunisia; Hu, Hungary; NY, New York; Is, Israel; Tx, Texas; Eg, Egypt; Chin, China; Ind, India; Rus, Russia; Rab, Rabensburg; CAR, Central African Republic; SA, South Africa, Ug, Uganda), 2 numbers for the isolation year (ex: 00 = 2000, 96 = 1996), and GenBank accession number. Sequences obtained from the 2 horse samples in Hungary 2008 are highlighted (rectangle). JEV, a close flavivirus, was used to root the phylogenetic tree.

similar to some results in human patients. In humans, CSF samples invariably show increased protein concentrations and pleocytosis, with a predominance of neutrophils[22] or lymphocytes.[3] In horses, the CSF was abnormal in most cases showing mononuclear pleocytosis with lymphocytic predominance, but in some cases, only a high protein concentration was found.[23] In 27% of the cases, the values were within reference ranges even during advanced disease.[24]

The antemortem diagnosis of acute WNV infection is based on the detection of specific IgM antibodies in serum, CSF or both by an ELISA test and an increase in IgG titers between acute-phase and convalescent sera. In IgM-positive cases, it is necessary to confirm the diagnosis by a neutralization test that is able to differentiate between closely related flaviviruses.[3,23] Cross reactions might have been caused by the tick-borne encephalitis virus, which

also is endemic in Hungary. At least 2 different types of serological tests were performed in each case and different serotests gave reliable, homogeneous results. RT-PCR with blood samples has low diagnostic sensitivity because of the low titers and short duration of viremia in horses and humans, and therefore, the virus may not be present in blood at the time of clinical manifestation of signs.[22,23]

With regard to the outbreak in Europe, there were 2 unique features: the causative agent was a sub-Saharan strain and the infections were not limited to a single geographic area.

This outbreak was caused by a lineage 2 WNV, which often is considered nonpathogenic for animals or humans. A lineage 1 strain also was shown to circulate in the same geographic area, and although this genetic line typically is responsible for outbreaks in Europe, it has

never caused an equine epidemic in Hungary.[8] The lineage 2 virus had been detected in 2004 and caused isolated encephalomyelitis cases in different species, but had never previously been associated with a severe equine outbreak. The strain sequenced during this epidemic in 2008 has diverged slightly from the virus isolated in 2004. International literature regarding WNV infections and disease caused by lineage 2 strains are somewhat conflicting. Some Southern African lineage 2 isolates were associated with single isolated cases of encephalitis in a human and a dog, and with fatal hepatitis in a human and death of an ostrich chick.[2] A recent South African retrospective study also reported the causative role of lineage 2 WNV strains in 7 cases of neurologic disease in horses.[19] Other experimental studies indicated that exposure of horses to the endemic Southern African strains of WNV was not associated with neurological disease.[25] Neurological disease was not detected in any of the horses included in the study, and 2 horses inoculated with a recent lineage 2 South African isolate of WNV showed no clinical signs of the disease after infection, virus was not detected in their blood.[25]

WNV had also re-emerged and caused equine cases in Italy in 2008. Phylogenetic analysis of the Italian isolates indicated that these viruses belonged to lineage 1 strains. Confirmed clinical cases occurred from the end of August to mid-October, similar to the cases in our study. Thirty-three horses showing clinical signs with a 15.1% fatality rate were reported from 3 regions along the Po River Delta, Italy.[9]

All WNV infections in Hungary were limited to the same wetland areas before August 2008, but in 2008, cases occurred in many parts of the country. Interestingly, the highest incidence occurred around the capital. Parallel with the chronology and geographic locations of equine infections, birds of prey died of WNV encephalitis and human cases also were diagnosed. According to the localization of the equine, avian, and human cases, Hungary experienced substantial northwestern spread of the pathogen. Six human cases were identified in Italy, but there was no significant increase in bird mortality.[9] The role of different bird species in transmission of the virus among different geographic locations and among rural, urban and suburban areas should be investigated.

Most lineage 2 WNV infections are not thought to be symptomatic in equids. Therefore, all infected horses may not have been identified. A serosurvey is ongoing to detect the extent of previous WNV infection among horses and birds. More equine encephalomyelitis cases may have occurred, but animals might have not been tested for the disease by local veterinarians because of lack of knowledge about WNV appearance in the region. This outbreak illustrates the possibility of the emergence and re-emergence of new viral infections in Hungary.

Footnotes

Acknowledgments

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