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Concurrent occurrence of human and equine West Nile virus infections in Central Anatolia, Turkey: the first evidence for circulation of lineage 1 viruses

Aykut Ozkul [a,*], Koray Ergunay [b], Aydan Koysuren [b], Feray Alkan [a], Ethem M. Arsava [c], Seda Tezcan [d], Gurol Emekdas [d], Sabri Hacioglu [a], Mahur Turan [a], Durdal Us [b]

[a] Department of Virology, Faculty of Veterinary Medicine, Ankara University, Irfan Bastug Cad. Diskapi, Ankara 06110, Turkey
[b] Department of Medical Microbiology, Faculty of Medicine, Hacettepe University, Ankara, Turkey
[c] Department of Neurology, Faculty of Medicine, Hacettepe University, Ankara, Turkey
[d] Department of Medical Microbiology, Mersin University Faculty of Medicine, Mersin, Turkey

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SUMMARY

Background: West Nile fever is an important zoonotic infection caused by West Nile virus (WNV), a member of the *Flaviviridae*. Previous serological data from Turkey suggest widespread WNV circulation. This report includes cases of human and equine WNV infections occurring concurrently, and manifesting as central nervous system infections, in two neighboring provinces of Central Anatolia, Turkey. A partial phylogenetic analysis of the causative virus is given for the first time.
Methods: The cases were reported in February (horses) and March (human). Symptoms of the disease were similar in the two species, characterized by neurological manifestations suggesting meningoencephalitis. Real-time/nested PCRs and commercial immunoassays and a plaque reduction neutralization assay were employed for the detection of viral RNA and specific antibodies, respectively.
Results: WNV RNAs were detected in buffy coat (horses) and cerebrospinal fluid (human) samples. Partial nucleotide sequences of the E-gene coding region revealed that the strains are closely related to viruses of lineage 1, clade 1a. Accompanying equine serosurveillance demonstrated WNV-specific antibodies in 31.6% of the samples.
Conclusions: This is the first report of acute WNV infections caused by lineage 1 strains from Turkey, in concordance with previous reports from some European and North African countries.

1. Introduction

West Nile virus (WNV) is an arthropod-borne flavivirus affecting a wide range of vertebrates, including birds and mammals.[1] The natural cycle of infection involves birds and mosquitoes of the *Culex* family; humans and horses are considered dead-end hosts. The transmission of WNV to susceptible individuals frequently requires the bites of infected culicine mosquitoes.[1,2] Although the majority of infections are mild or asymptomatic, WNV may cause outbreaks of febrile disease and encephalitis in humans and horses.[2] While less than 1% of human cases experience severe disease, up to 90% of symptomatic cases in horses result in a neurological disease with case-fatality rates of 30–40%.[2–5] Initially identified in tropical Africa, WNV infection has been found in northern Africa, Israel, India, Australia, and Europe, and has progressively spread in the Americas since 1999.[2,6] Since 1994, the virus has caused frequent outbreaks of severe neuroinvasive disease in humans and horses in Europe and the Mediterranean Basin.[6]

In Turkey, no reports of symptomatic WNV-related disease in humans or animals were available until 2009, despite published reports with compelling evidence of widespread virus circulation in different geographical areas.[7] Cases of WNV-related central nervous system (CNS) diseases have since been reported, as well as an outbreak in Western Anatolia in 2010.[7,8] However, diagnoses were confirmed via the detection of specific antibodies against WNV, and viral RNA detection and characterization have so far been unsuccessful. In this study, we report the concurrent occurrence of WNV infections in two cases of equine and one case of human acute disease, during the winter of 2011. The cases presented with CNS involvement. A successful virus isolation and partial genetic characterization was performed for the first time in Turkey.

* Corresponding author.
E-mail address: ozkul@ankara.edu.tr (A. Ozkul).

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2. Materials and methods

2.1. Equine cases

Neurological symptoms including muscle weakness in the rear limbs, muscular tremor, irritability, postural instability, and movement disorders were identified in two purebred Arabian foals (ID# 82 and ID# 88) after initial flu-like symptoms including slight fever, loss of appetite, and chills. This was recorded in a stable in Eskisehir Province (39°24′N-31°02′E) on January 18, 2011 (Figure 1). The animals were 8 and 9 months old when the disease was noted. Serum and plasma samples were tested for equine CNS viruses including equine herpes virus (EHV)-1, EHV-2, EHV-4, and EHV-5, and WNV nucleic acids by PCR. The foals had made a full recovery approximately 1 week after disease onset. Following this episode, a total of 180 randomly-selected serum samples were collected (on March 5, 2011) from horses of the same herd, regardless of age or gender, for serological assessment of WNV exposure within the herd.

2.2. Human case

A 61-year old woman living in uptown Ankara (39°56′N-32°52′E; Figure 1) with no underlying disease other than hypertension was admitted to Hacettepe University Hospital with a preliminary diagnosis of viral encephalitis on March 14, 2011. Her complaints had started 1 week prior to admission with fever and flu-like symptoms.

Upon admission, she was confused, uncooperative, and disoriented, with speech fragmentation and mild paraphasic errors. Furthermore, myoclonic jerks and a resting tremor of the upper extremities were noted, without signs of meningeal irritation. No maculopapular or any form of rash was observed during the disease course. Laboratory evaluation revealed mild leukocytosis (12×10^9/l) and elevated cerebrospinal fluid (CSF) protein (67.81 mg/dl), without pleocytosis or a decrease in glucose levels. An electroencephalogram (EEG) showed diffuse slowing of the background rhythm, with sharp slow waves in the temporal, parietal, and occipital regions of the right hemisphere. Magnetic resonance imaging (MRI) was within normal limits except for an incidental right occipital meningioma. Initial serum–CSF samples and follow-up serum–CSF pairs taken on days 9 and 20 revealed no evidence of bacterial, mycobacterial, or fungal infections by culture or rapid antigen assays. All samples were also negative in PCR for herpes simplex virus (HSV) type 1/2 and *Mycobacterium tuberculosis*. The CSF immunoglobulin index and pathogen-specific globulin indices for HSV, measles, mumps, and rubella were also within normal limits. The serum–CSF pairs were evaluated for WNV and Toscana virus (TOSV) RNA and serology, as detailed below. The patient was administered levetiracetam, acyclovir, and ceftriaxone. Her neurological condition started to improve within a week, and she demonstrated full cooperation and orientation at the time of discharge, approximately 3 weeks after admission. She did not have any history of mosquito, tick, or sandfly exposure, transfusion, outdoor activity, or horseback riding, and had never been vaccinated for flavivirus infections. She resided in Ankara Province and had not travelled for the last 7–8 months.

2.3. Nucleic acid tests (NAT) and confirmatory assays

Initially, WNV RNA was sought in all samples via an in-house real-time PCR assay, as described previously.[9] A High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Germany) was used for the purification of nucleic acids, and a Fermentas RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Japan) was used for cDNA synthesis; kits were used in accordance with the manufacturer's instructions. A nested PCR assay was employed for a 240-bp final product in the E-protein coding region of the genomic RNA, as described previously.[10] Dilutions of extracted RNA from culture-adapted WNV (strain NY99-4132) were used as positive controls in the PCR assays. The second-round DNA products of the nested PCR were cleaned via PureLink PCR Purification Kit (Invitrogen, Netherlands) and subjected to nucleotide sequencing. Neighbor-joining (NJ) analyses of the sequences were carried out for phylogenetic evaluation using CLC Main Workbench v5.5 (CLCBio, Denmark). NAT for TOSV and other phleboviruses in serum and CSF samples from the human patient were performed using generic and specific TOSV primers in a nested PCR reaction, as described previously.[11]

Buffy coats remaining from the blood samples of the sick horses and CSF from the patient were inoculated onto Vero cell (ATCC CCL81) monolayers for virus isolation. Daily microscopic examinations to monitor WNV-indicative cytopathic effects were performed for 7–9 days post-inoculation. Samples were serially passaged in Vero cells, and culture fluids (processed as explained above) were subjected to the nested PCR and subsequent sequencing, after each culture passage.

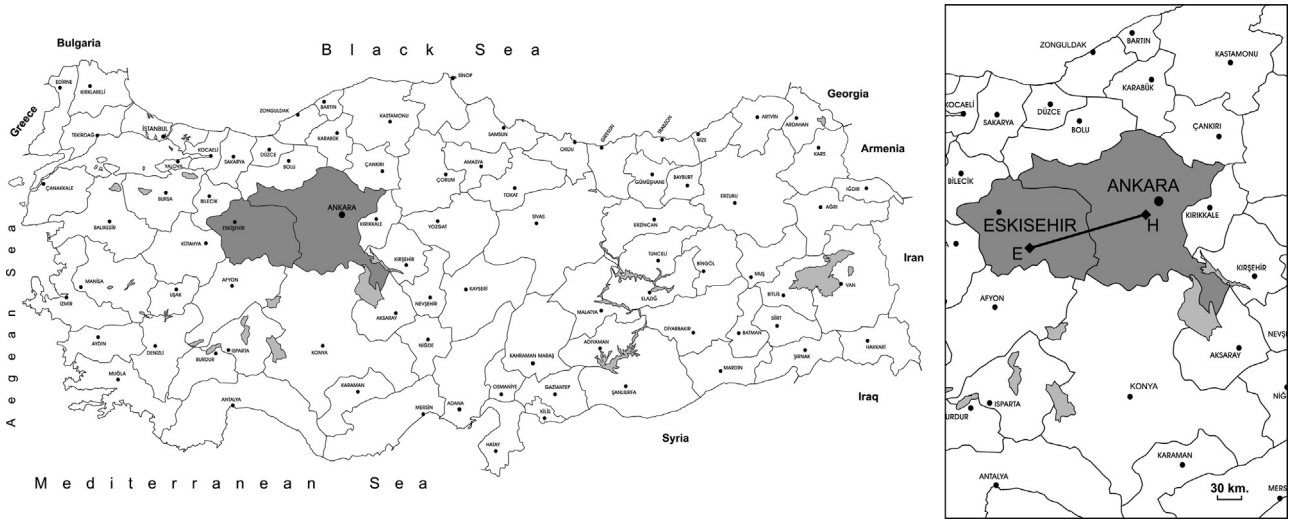

Figure 1. Map showing the areas where human and equine cases of WNV were detected. The distance between the two locations is indicated with a bar representing about 150 km. 'E' indicates equine case locations, 'H' indicates the human case location.

2.4. Serological screening

For the evaluation of patient samples for WNV and TOSV immunoglobulins, commercial assays (West Nile virus IgM and IgG IIFT and ELISA, Toscana Virus IgM and IgG IIFT; Euroimmun, Germany) were employed as directed by the manufacturer. Patient samples reactive for WNV immunoglobulins were further tested via plaque reduction neutralization test (PRNT), as described previously.[12] PRNT was employed for screening and specificity confirmation of WNV immunoglobulins in the horse sera. Samples with neutralizing antibodies against WNV were re-evaluated via PRNT in serial two-fold serum dilutions starting from 1:5 to assess the mean antibody titer.

3. Results

3.1. Virological studies

In the human case, all samples were negative in serological assays and generic PCR for TOSV and phleboviruses. However, the CSF sample taken on day 1 was reactive for WNV RNA via the initially performed real-time PCR and subsequent nested PCR. CSF positive for viral RNA lacked specific IgM, however IgM in the paired serum was borderline. While all of the subsequent CSF samples were negative for viral RNA and immunoglobulins, subsequent serum samples were observed as reactive for WNV IgG and were further confirmed for specificity via PRNT in 1:20 dilution.

In the horse cases, WNV RNA was detectable in blood samples using both the initial real-time PCR and follow-up nested PCR. The samples were interpreted as negative by PCR for EHVs.

All human and equine samples with WNV RNA were inoculated onto Vero cells. While two WNVs were isolated from buffy coat samples of pre-diagnosed horses, no virus was recovered at the end of three blind passages of CSF from the human case. The virus titer was higher (median tissue culture infective dose ($TCID_{50}$) 10^3/0.1 ml) in one horse (ID# 82) than in the other (ID# 88) following initial isolation. Virus isolates were further characterized using a nested PCR.

Nucleotide sequencing from the sample amplicons and the corresponding culture supernatants displayed identical sequences, which have been deposited in GenBank (accession numbers **JN828805** and **JN828806**). Analysis of nucleotide sequences of both viruses indicated 99.1% identity with only two nucleotide substitutions. Phylogenetic analysis (NJ) revealed that the two viruses detected in the two horses and human were closely related to lineage 1 WNV strains, particularly to clade 1a (Figure 2). Despite repeated trials, the length of the sequence obtained from the human case remained around 118 bases and thus could not be submitted to GenBank.

3.2. Serosurveillance in horses

PRNT revealed that 57 (31.6%) of 180 horses randomly sampled had specific antibodies against WNV. Of the reactive serum samples, 12 (21%) were found to have an antibody titer of >1:40. The majority (79%) of the remaining serum samples showed antibody titers between 1:10 and 1:20.

3.3. Features of the locations where WNV infections were detected

The two centers in which the WNV-related meningoencephalitis cases in the human and in the horses were detected are located in the mid-Anatolia region of Turkey, approximately 150 km apart (Figure 1). According to the Thornthwaite classification, this region has a semi-arid climate type.[13] Its most important characteristic is contrasting severe winters and hot summers with low rainfall levels. The official meteorological service reports average temperatures (min/max) for February and March in the region of 1.4 °C (−2.9/6.4) and 5.2 °C (−0.5/11.6), respectively, based on data obtained from 1975–2011 measurements (URL: http://www.dmi.gov.tr/veridegerlendirme/il-ve-ilceler-istatistik.aspx?m=ESKISEHIR).

4. Discussion

Continuous but low-level WNV circulation has been reported from various countries of continental Europe.[14] In 2011, the European Centre for Disease Prevention and Control (ECDC) and the World Organisation for Animal Health (OIE) declared a total of 86 human cases and 14 cases in horses.[15] In Turkey, a country neighboring the European Union states, previous serosurveillance data have indicated human WNV exposure in various regions, including Central, Western (Aegean coast), Southern (Mediterranean coast), and Southeastern Anatolia.[7] Confirmed seroprevalence rates of 0.56–0.8% have been demonstrated in Central Anatolia in asymptomatic blood donors.[16,17] Moreover, neutralizing WNV antibodies have been demonstrated in a variety of mammalian species, including horses.[18] However, despite evidence of virus circulation, there are very few documented cases of WNV infection reported from Turkey.[7,8,19–22] Our findings represent the first WNV sequence data obtained from human CNS infections and the first report of acute WNV infection in horses.

Currently, a limited number of reports of acute WNV infection and WNV-associated neurological disease from Turkey are available. Probable WNV-associated encephalitis in humans was previously suggested in residents of Ankara Province in 2009, and the diagnosis was further confirmed via PRNT in a 62-year-old woman.[19,20] The symptoms observed in these patients were similar to those of the current case, in whom symptoms consistent with relatively mild encephalitis started abruptly following an unspecified febrile episode; the patient responded favorably to supportive therapy and there were no neurological sequelae. WNV-associated encephalitis was previously identified in Ankara Province in a 62-year-old woman in 2009,[6] as well as in two men, aged 56 and 61 years, in 2010.[21] The symptoms observed in these patients were high fever, headache, malaise, confusion, and altered consciousness, without focal neurological manifestations. CSF demonstrated lymphocytic pleocytosis and increased protein levels and the presence of specific IgM. No significant findings on EEG or neuroimaging were observed, and all cases responded favorably to supportive therapy without residual neurological sequelae. An outbreak of acute WNV infection emerged in 2010 in Western Anatolia and involved 12 serologically confirmed and 35 probable cases.[8] Forty of the 47 cases displayed West Nile disease with the most common symptoms being fever (100%), headache (85%), nausea/vomiting (75%), and altered consciousness (57.5%). However, CSF examination was performed in a limited number of cases and no data regarding neurological evaluation or nucleic acid assays were available. In 2011, a fatal case of serologically proven West Nile disease with tremors and impaired consciousness was also reported from Ankara Province.[22] A distinctive clinical feature of our patient's presentation was the presence of myoclonic jerks and a resting tremor of the upper extremities. Symptoms or laboratory evidence of encephalitis is observed in about 60% of persons with West Nile disease,[23] and tremors and myoclonus, where primarily affecting the upper extremities and/or facial muscles, have been reported in less than a third of cases presenting with encephalitis.[23–25] Thus, cases of CNS infections of presumed viral etiology presenting with atypical symptoms must also be evaluated for WNV-related disease.

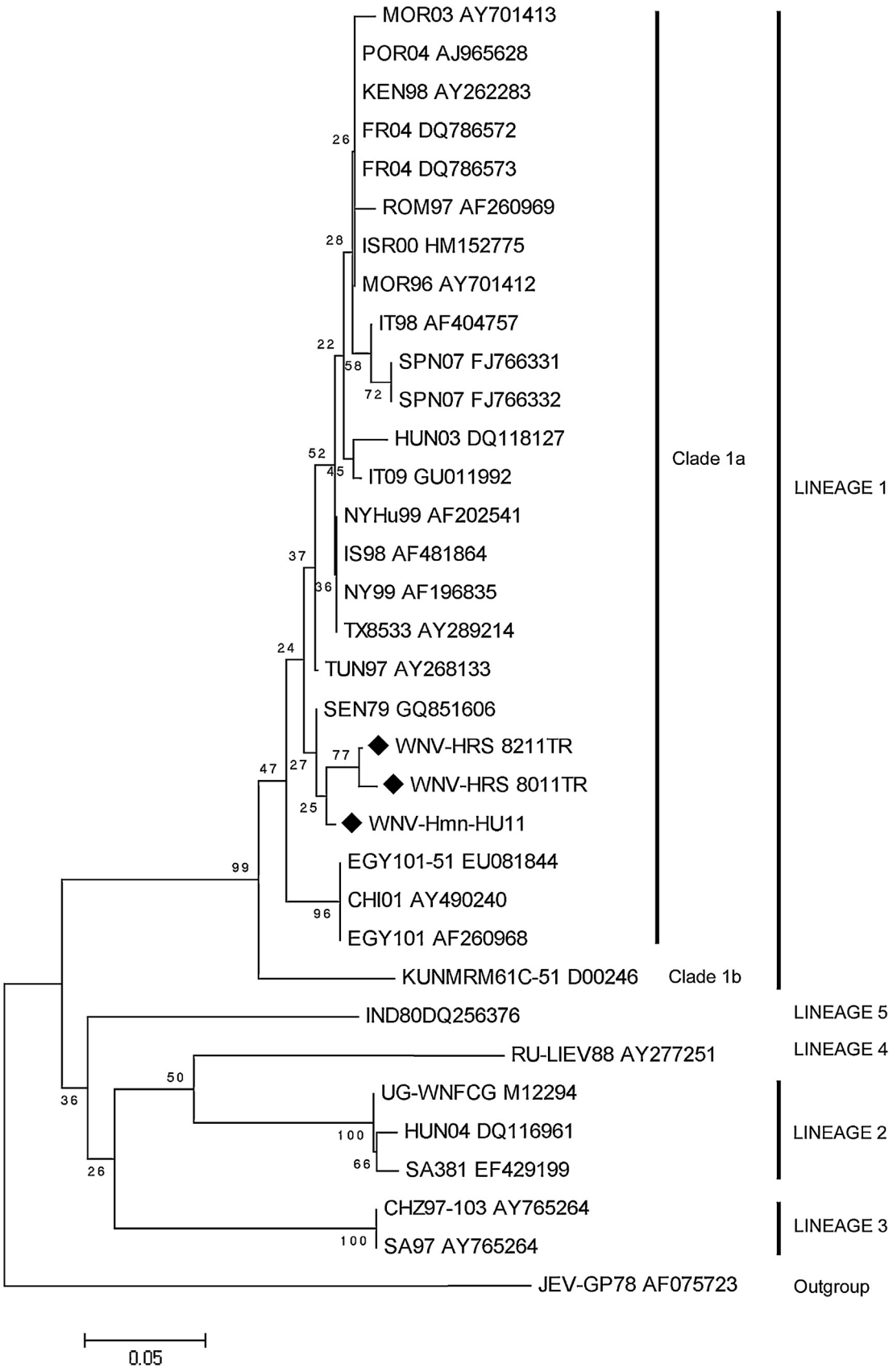

Figure 2. Phylogenetic analysis of WNV detected in two horses in Turkey. The tree was generated based on the partial sequence of the E protein coding region of the genome by neighbor-joining (NJ) analysis with 1000 replicates of bootstrap confidence level in CLCBio V5.2, Main Workbench Software (Denmark). Viruses used for the NJ analysis are described by abbreviations for location/isolation date, followed by the GenBank accession number. Local WNV isolates in the tree are indicated with bold diamonds. JEV GP78 represents outgroup viruses. The numbers at the nodes indicate bootstrapping values. The bar represents nucleotide substitutions per position (118 nt sequence from the human case (WNV-Hmn-HU11TR):GTCCACCGTGAGTGGTTCATGGACCTCAACCTCCCCTGGAGCAGTGCTGGAAGTACTGTGTGGAGGAACAGAGAGACGTTAATGGAGTTTGAGGAACCACACGC CACGAAGCAGTCTG).

The first descriptive equine cases related to WNV were reported in the USA soon after the first occurrence of WNV disease in humans in 1999.[26] The most frequent symptom noted in those cases was ataxia (present in 85%), and about 50% of affected animals also demonstrated limb weakness, recumbency, and muscle fasciculation.[26] The clinical findings in the horses of the current study are in accordance with those previously described.[26,27]

The most critical observation in the current study is the seasonal occurrence of the clinical disease. Symptomatic human and equine infections with detectable viral RNA in CSF and buffy coat, respectively, were identified in February and March, unfavorable seasons for WNV transmission due to the diminished activity of mosquito species. Moreover, the fairly severe winters of the Central Anatolia region would have been expected to prevent transmission via vectors.[13] Although no evidence for the route of WNV transmission could be identified for our cases, the disease can be speculated to emerge due to overwintering viruses, involving previously identified mechanisms of mosquito hibernation. It is well documented that WNV may biologically persist and evolve in hibernating mosquitoes.[28,29] A study carried out by Clements demonstrated that female *Culex pipiens pipiens* mosquitoes removed from hibernation could fly 4300 meters on average, with a maximum range of 8674 meters, and were able to blood feed when appropriate if conditions and hosts were available.[30] A similar mechanism might be responsible for the emergence of the human and horse infections, as the locations of emergence were geographically related. Since hibernating blood-fed Culex mosquitoes may contain high amounts of WNV, removal from hibernation and flight over the above-mentioned distances could result in the efficient transfer of the virus to susceptible dead-end hosts.[29–31] Moreover, large capacity stables (e.g., organized stud farms) are considered to provide good shelters for birds and mosquitoes during the winter season, and if environmental conditions (heat, humidity, and nutritional resources) within the stables are sufficient for the physiological stabilization of the insects, hibernation may not be required for survival.[29–31] An entomologic survey has already been launched by our group to investigate potential routes for WNV exposure in the region, and preliminary findings indicate the abundance of *Culex pipiens pipiens* mosquitoes in the region. However, the exact route of WNV transmission in our cases remains obscure and needs to be elucidated.

Five genetic lineages of WNV have been identified, the major two being lineages 1 and 2.[32] The majority of the strains responsible for the European and Mediterranean Basin outbreaks belong to lineage 1.[14] However, recent data also suggest the circulation of and symptomatic infections due to lineage 2 strains as well.[14,32] In Greece, a north-western neighbor of Turkey, a large WNV outbreak occurred in 2010, followed by another in 2011, resulting in over 250 cases of neuroinvasive disease with 15% fatality.[33,34] WNV lineage 2 sequences were obtained from blood donors, Culex mosquitoes, and sentinel animals, and the WNV strain displayed high genetic similarity to the goshawk-Hungary/04 WNV strain.[34,35] Partial sequences obtained from human and equine infections in this study demonstrated that the causative virus strains are grouped in lineage 1, clade 1a, and closely related to North African strains (Figure 2). The origin and routes of introduction of WNV lineage 1 strains in Turkey are not yet clear, although several mechanisms including spillover from Eastern Mediterranean regions can be proposed. Nevertheless, the presence and distribution of lineage 1 as well as potential activity of lineage 2 strains in Turkey require further data from genetic analyses of other strains from various parts of Turkey. Studies are under way to sequence the whole genome of the present isolates, which will provide more information about the origin of the strains in circulation.

In conclusion, we have identified WNV RNA in acute CNS infections in humans and horses for the first time in Turkey. WNV must be considered in the viral etiology of neuroinvasive infections in both species. Given that the current study further reports the occurrence of WNV disease in winter, viral transmission dynamics and epidemiological features need to be elucidated. WNV has been responsible for human, equine, and avian epidemic outbreaks in many regions of the world and remains a serious health problem with no protective vaccine or effective antiviral treatment. Moreover, the certain predictability of its possible emergence in new territories is only now beginning to be realized. Turkey, in common with several other regions of Europe and Asia, was a region where convincing evidence originating from virus isolation or nucleic acid detection in human or animal specimens had been lacking. Based on RT-PCR sequencing, serological investigation, and virus isolation studies in horses and one human case, this study finally confirms that WNV is indigenous in Turkey.

Acknowledgements

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Ethical approval: The current study was performed as part of public health practice. The human and animal cases were studied and are presented here with the official permission of the individual and the animals' owners.

Conflict of interest: We have no competing interests to declare.

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